Porcine Milk Fat Globule Membrane Proteins that Bind to F4ac Fimbriae and prevent attachment of *Escherichia coli* to enterocytes

A Thesis Submitted to the College of

Graduate Studies and Research in

Partial Fulfillment of the Requirements

for the Degree of Master of Science in

the Department of Veterinary Pathology

University of Saskatchewan

Saskatoon, Saskatchewan

By

PREDRAG NOVAKOVIC

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ABSTRACT

Post-weaning diarrhea (PWD) in pigs is caused by enterotoxigenic *Escherichia coli* (ETEC) which is often controlled by antibiotic feed supplements with the potential risk of generation of antibiotic resistant bacteria. Prevention of attachment of F4ac positive ETEC to intestinal mucosa may potentially be used as one of the antibiotic-free strategies for the control of this disease. F4ac-positive ETEC has been reported to bind to fat globule membranes in porcine milk.

In this study, we used an affinity chromatography technique to identify individual milk fat globule membrane (MFGM) proteins that bind to F4ac fimbriae. An affinity column with covalently coupled F4ac fimbriae to activated Sepharose was created and the following F4ac-binding proteins were isolated from the porcine MFGM: lactadherin, butyrophilin, adipophilin, acyl-CoA synthetase 3, fatty acid binding protein 3, and xanthine dehydrogenase.

Selected individual proteins of porcine MFGM, namely: xanthine dehydrogenase, butyrophilin, lactadherin, and fatty acid binding protein were isolated and tested for their inhibitory effects against attachment of F4ac positive ETEC and of F4ac fimbriae to either a small intestinal cell line (IPEC-J2) or primary porcine enterocytes by competitive ELISA. All of these proteins, except xanthine dehydrogenase, decreased attachment of F4ac positive *E. coli* and F4ac fimbriae to the intestinal cell line or primary enterocytes in a dose-dependent manner. Lactadherin demonstrated the strongest inhibitory effect on F4ac positive *E. coli* adherence to porcine enterocytes and the most potent interaction with F4ac fimbriae.

ACKNOWLEDGMENTS

The success of the research project is not possible without strong teamwork. I was fortunate that I had the opportunity to do my MSc project collaborating with many outstanding researchers. Each of them, more or less, gave a unique contribution for achieving the final goal, results for publication. I am heartily grateful to each of them for what I learned. Each method, technical, administrative or personal advice had a profound impact on my overall progress, so my understanding, perception and knowledge on the objectives of my research were constantly expanding and improving qualitatively.

Particularly, I want to express my profound appreciation to my supervisor, Dr. Elemir Simko, whose generosity, support and incredible memory, 2 years after our initial contact, had lead me to Canada, and to this project. I also want to thank Dr. Beverly Kidney and Dr. Matthew E. Loewen, for their helpful assistance as members of my committee. I specially thank Dr. Charavaryamath (Chandru) Chandrashekhar for his valuable suggestions throughout the various experiments. My thanks are extended to Betty Lockerbie for the technical support, Dr. Volker Gerdts, and Dr. Wojciech Dawicki for sharing their expertise in science. I would like to give a special thanks to Tyler Moss and Sandy Mayes, who were always willing to help with patience and kindness. I thank all the graduate students in this department, especially Keyvan Amini, and Rodolfo Nallar for their sincere friendship and encouragement.

Finally, I want to express my deepest appreciation and gratitude to all the faculty, staff and students in the Department of Veterinary Pathology with words from a great Isaac Newton:

“If I have seen further than others, it is by standing upon the shoulders of giants”

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LIST OF ABBREVIATIONS

Abbreviation	
1D	one-dimensional
2D	two-dimensional
ACS3	acyl-CoA synthetase 3
ADPH	adipophilin
AIDA	adhesin involved in diffuse adherence
BSA	bovine serum albumin
BTN	butyrophilin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CV	column volume
Da	daltons
DTT	dithiothreitol
E. coli	*Escherichia coli*
EAST1	enteroaggregative *E. coli* heat-stable enterotoxin
ED	edema disease
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EPEC	enteropathogenic *E. coli*
ER	endoplasmic reticulum
ETEC	enterotoxigenic *E. coli*
FABP	fatty acid binding protein
FAD	flavin adenine dinucleotide

HBGAs	H-2 histo-blood group antigen
Ig	immunoglobulin
LAD	lactadherin
nanoLC-MS/MS	nanoliquid chromatography mass spectrometry/mass spectrometry
LF	lactoferrin
LPS	lipopolysaccharide
LT	heat labile enterotoxin
MFG-E8	milk fat globule-epidermal growth factor-factor 8
MFGM	milk fat globule membrane
MW	molecular weight
NK cells	natural killer cells
PAGE	polyacrylamide gel electrophoresis
PAS 6/7	periodic acid Schiff 6/7
pI	isoelectric point
PVDF	polyvinylidene fluoride
PWD	post-weaning diarrhea
PRRS	porcine respiratory and reproductive syndrome
RGD	arginine-glycine-aspartic acid recognition sequence
SDS	sodium dodecyl sulfate
ST	heat-stable enterotoxin
XDH/XO	xanthine dehydrogenase/oxidase

1 GENERAL INTRODUCTION

In 2000, the World Health Organization announced the report on infectious diseases entitled "Overcoming antimicrobial resistance" and introduced the world to a new phenomenon that threatened to shake the foundations of modern medicine. The 3rd chapter of this report begins with an anonymous quote:

"The History of Medicine:

2000 B.C. Here, eat this root.

1000 A.D. That root is heathen. Here, say this prayer.

1850 A.D. That prayer is superstition. Here, drink this potion.

1920 A.D. That potion is snake oil. Here, swallow this pill.

1945 A.D. That pill is ineffective. Here, take this penicillin.

1955 A.D. Oops . . . bugs mutated. Here, take this tetracycline.

1960-1999 A.D. 39 more "oops.". . . Here, take this more powerful antibiotic.

2000 A.D. The bugs have won! Here, eat this root" [1].

This humorous illustration poses a simplistic but realistic presentation of the long human history to fight infectious disease. Nevertheless, infectious diseases continue to be the principal cause of morbidity and mortality on our planet. There are many reasons for this, but the phenomenon of antimicrobial resistance, has allowed many strains of bacteria to become resistant to almost all available drugs. This has become a leading global public health concern both in human and veterinary medicine [2].

The antimicrobial agents in veterinary medicine are used as growth promoters, prophylactics and as therapeutic agents. When used to promote growth, low doses of antimicrobial agents are introduced into the feed of healthy animals for a long period of time. For prophylactic purposes they are used, at either low doses or therapeutic doses, to prevent infectious disease or control the dissemination of already diagnosed infectious disease within a group of animals. In addition, medicated feed is used worldwide for treatment of sick herds. The swine and poultry industry are especially affected with this practice, because individual sick animals are difficult to treat [3]. All of these approaches may result in the overuse or misuse of antibiotics and development of bacterial resistance [4].

The emergence of antimicrobial resistance of enterotoxigenic *E. coli* (ETEC), the cause of porcine post-weaning diarrhea (PWD), has become the focus of attention because of the increased incidence of this disease in the modern swine industry [5]. The *E. coli* isolates are multiresistant to a range of antimicrobials including oral and injectable antibiotics, especially those frequently used in treatment against PWD, such as apramycin, neomycin, spectinomycin and trimethoprimsulfonamide [6]. The ban on the use of antibiotics as growth promoters (avoparcin, bacitracin, spiromycin, tylosin, and virginiamycin) in European countries resulted in a higher incidence of PWD. The consequence of this ban was the increase in therapeutic use of aminoglycosides, macrolides, and lincosamides, which in consequence has led to development of antimicrobial resistances in *E. coli* [7]. Therefore, in order to stop antimicrobial resistance in enterotoxigenic *E. coli* associated with PWD, many alternative strategies for prevention and control have been examined to this point.

The aim of studies presented in this thesis was to investigate proteins of porcine milk fat globule membrane (MFGM), which was previously demonstrated to be able to interact [8] and inhibit attachment of ETEC to the intestinal brush border [9]. The protective function of colostrum and milk immunoglobulins is well characterized and indisputable. However, both colostrum and milk also contain a myriad of non-immunoglobulin related substances potentially involved in protection of neonates against infectious diseases. Our research group isolated from porcine skim milk several F4-fimbrial-binding proteins, two of which, namely lactadherin and heart fatty acid binding protein, are major MFGM proteins in several species [10]. Subsequently, it was demonstrated that lactadherin interfered with attachment of F4-positive ETEC to porcine small intestinal villi *ex vivo* [11]. Since MFGM contains many proteins, it is reasonable to hypothesize that lactadherin is not the only one that interacts with F4-fimbriae. Accordingly, the first objective of this study was to investigate F4-fimbrial-binding proteins in porcine MFGM by affinity chromatography. The second objective was to determine inhibitory effects of F4-fimbrial-binding proteins in porcine MFGM on attachment of F4ac ETEC to primary porcine enterocytes and porcine small intestinal epithelial cell line (IPEC-J2) using ELISA.

2 LITERATURE REVIEW

2.1 Post-weaning diarrhea in pigs

It's been almost a half of century, since Sojka (1965) started to study porcine post-weaning diarrhea (PWD), but this disease still causes significant economic losses in the modern swine industry. Many studies have investigated the etiology [12], epidemiology [13], pathogenesis [14] and prevention [5] of porcine PWD, yet it seems that new and more effective strategies for prevention and control of PWD are needed in intense swine production.

Porcine PWD, also known as post-weaning enteric colibacillosis, is infectious diarrhea primarily caused by enterotoxigenic *Escherichia coli* (ETEC). O149 serogroup is by far the most dominant ETEC involved in pathogenesis of PWD [15]. Among various strains of ETEC, only two can be found worldwide as a cause of PWD, namely F4 and F18. They are identified in 92.7% of all ETEC related cases of PWD [12]. Another pathotype of *E. coli* involved in the etiology of this disease, to a lesser degree, is enteropathogenic *E. coli* [5].

During the first week after weaning, the piglets are most susceptible to PWD due to many stress factors involved (stress of weaning, dietary changes) and lack of antibodies and potentially other protective substances from a sow's milk. The strains of ETEC (F4 and F18) also have a significant effect on the timing of PWD outbreaks. The F4 strain can cause neonatal, preweaning and postweaning diarrhea; the postweaning diarrhea caused by F4 strain occurs most often within a few days after weaning. On the other hand, F18 strain more often causes PWD between 5 and 14 days after weaning or even at introduction of pigs to finishing herds [15].

The typical clinical signs of PWD include sudden onset of watery (secretory) diarrhea that begins 16-36 hours after infection, lethargy, and cyanosis of the tip of the nose, the ears, and the abdomen [14]. If prolonged, dehydration and depression due to diarrhea can result in severe growth retardation. Sudden death can also occur occasionally during the first few days after weaning [15].

The PWD is a disease characterized by high morbidity and variable mortality. Gross lesions in animals that died due to acute or subacute PWD may include: congested, flaccid small intestines affected sometimes by acute catarrhal enteritis and gastric venous infarction. Microscopically, the most prominent change is the presence of small clumps or even continuous layers of ETEC (isolated in almost pure culture) attached to the surface of enterocytes on villi of the small intestine [16].

2.1.1 Pathogenesis

There are two distinct steps in development of porcine post-weaning diarrhea caused by ETEC, namely, colonization and production of enterotoxins. Colonization is the first step in pathogenesis and starts with attachment of previously ingested ETEC from contaminated feed, water or environment, to small intestine. The proliferation of a sufficient number of bacteria on the mid-jejunal and ileal intestinal surface is the essential requirement for development of PWD [5]. Adhesion of ETEC to the cell membrane of enterocytes is mediated by the interaction between fimbriae and specific receptors on the enterocytes [17]. Intestinal receptors for F4 fimbriae are present in susceptible pigs from birth to adult age. For this reason, F4 positive ETEC can cause diarrhea in neonatal, postweaning and even finisher pigs. On the other hand, the

F18 strain of ETEC cause PWD only in pigs older than 3 weeks [5], because F18 receptors are expressed in piglets after 20 days of age [14].

The second step in the pathogenesis of PWD is the development of secretory diarrhea induced by enterotoxins produced by colonized ETEC on the small intestinal surface. The enterotoxins cause the change in the water and electrolyte flux of the small intestine resulting in excess fluid in the large intestine, and diarrhea. The F4 strain of ETEC produces heat-labile (LT), heat-stable (ST), and EAST 1 enterotoxins [14].

Heat-labile enterotoxin has 5 B-subunits and one A-subunit (A1 and A2 fragments). B-subunits are responsible for binding to GM_1 gangliosides receptors, while A-subunit (A1) has enzymatic activity (ADP–ribosyl transferase) that stimulates production of cyclic adenosine monophosphate (cAMP). After LT enterotoxin is internalized in the cell by receptor-mediated endocytosis, and undergoes retrograde transport through the endoplasmic reticulum, A1 fragment is released into the cytosol. The A1 fragment moves to the cytoplasmic face of the basolateral membrane and transfers the ADP–ribose moiety from nicotinamide adenine dinucleotide (NAD) to the Gs protein that stimulates adenylate cyclase. The end result is an excessive level of cAMP, as ATP is converted by adenylate cyclase to cAMP [5]. Cyclic AMP acts via stimulation of A kinase with direct phosphorylation and activation of the major chloride channel in intestinal epithelial cells, the cystic fibrosis transmembrane conductance regulator (CFTR) [18]. The active secretion of Cl^- ions by chloride channels on the apical membrane of the cell causes a decrease in the levels of Cl^- ions in the cell. This electrochemical gradient generated by the exit of Cl^- ions drives the Na^+-$2Cl^-$-K^+ cotransporter on the basolateral membrane to transport more Cl^- ions into the cell [19]. Additionally, the increased levels of cAMP, active apical chloride secretion and increased sodium entry into the cell by the Na^+-$2Cl^-$-K^+ cotransporter, increases the activity of

the Na^+-K^+ ATPase. This pump helps maintain the resting membrane potential by intensively pumping 3 sodium ions out and 2 potassuim ions into the cell, regenerating the ion gradient. This allows the continued increased transport of Cl^- ions through the Na^+ -$2Cl^-$ -K^+ cotransporter into the cell [20]. All of these activities together contribute to a significant increase in chloride secretion, which creates the osmotic drive for the massive loss of water into the intestinal lumen.

Heat-stabile enterotoxin, STa binds guanylyl cyclase C protein, which results in activation of guanylate cyclase and increased levels of cyclic guanosine monophosphate (cGMP) inside enterocytes. Production of cyclic guanosine monophosphate (cGMP) facilitates the passage of chloride out of the cell and reducing the absorption of water from the intestine by activating chloride channel, CFTR.

LT and STa enterotoxins both have effect on the activity of the enteric nervous system by stimulating serotonin (5-HT) release from intestinal enterochromaffin cells [18]. Excessive levels of serotonin can either directly stimulate intestinal epithelial cell Cl^- ions secretion or indirectly by initiating release of neurotransmitters such as acetylcholine (ACh), VIP, and nitric oxide (NO) from myenteric and submucosal plexus. The latter effect of serotonin will enhanced the blood flow into the intestine due the vasodilatation induced by VIP and NO, which will additionally contribute in maintaining high secretory rates during diarrhea [20].

EAST 1 is a commonly found enterotoxin in the F4 strain of ETEC pigs with diarrhea; it shares considerable amino acid sequence homology with the STa toxin. Hence, it is believed that EAST 1 causes diarrhea by the same mechanism as STa [21]. However, purified EAST1 from enteroaggregative *E. coli* did not show biological activity in the suckling mouse assay that detects STa activity [22]. In addition to that, it is demonstrated that among all strains of different pathotypes of enterotoxigenic *E. coli*, when inoculated into newborn colostrum-deprived pigs,

only EAST1 positive strain did not induce diarrhea [23]. Therefore, further investigations are needed to explain its role in ETEC associated with PWD.

2.1.2 The fimbriae of ETEC

In 1955, Duguid demonstrated that the agglutinating properties of *E. coli* are associated with the long non-flagellar filamentous appendages on the bacterial surface. They were called fimbriae [24].

A fimbria (plural fimbriae) is a long proteinaceous appendage radiating from the surface of the bacterium to a length of 0.5 to 1.5 nm [17]. The numbers of fimbriae can range from 100 to 1000 per bacterium, and their distribution is peritrichous (around the entire surface of bacteria). In the past, terms fimbriae and pili were used interchangeably, but morphologically and functionally they are different. Fimbriae represent the group of proteinaceous surface appendages, which are thin (diameter 2-4 nm) and flexible with a poorly defined structure, while the pili are rigid morphological structures with a diameter of 7-8 nm and an axial hole [17]. Many types of fimbriae have been reported for *E. coli,* for example F4 [5], F5 [25], F6 [26], F18 [5], F41[27]. However, only F4 and F18 positive ETEC strains are isolated from a majority of the cases of PWD in pigs. In addition, fimbrial adhesin called AIDA-1 (adhesin involved in diffuse adherence) has been identified in certain strains of ETEC associated with PWD [23].

2.1.2.1 F4 fimbriae

In 1961 Ørskov described the F4 fimbria and called it K88, according to capsular (K) antigen [28]. In 1966, Stirm isolated K88 by heating (60^{o} C) and homogenizing bacterial culture and demonstrated that it was a protein. Electron microscopy showed that K88 was not a capsular antigen, but a flexible fimbrial structure with a diameter of 2.1 nm [29]. Therefore, the nomenclature changed from the K (Kapsula) to F (Fimbria) designation.

The F4 fimbria is a protein with a molecular weight of approximately 27 kDa, composed of many repeating major subunits known as FaeG and with a small number of minor subunits scattered throughout the structure. The minor subunits are FaeC, FaeD, FaeE, FaeF, FaeH, FaeI and FaeJ. While the major FaeG subunit is only responsible for its adhesive functions, minor subunits are involved in the process of fimbrial synthesis [30-31]. Based on differences in primary amino acid sequences of the major FaeG subunit, F4 strains are separated into 3 serological variants, F4ab, F4ac and F4ad. The “a” determinant refers to an antigenically conserved segment and “b”, “c”, and “d” determinants to variable antigenic segments of F4 fimbria [17].

There are 6 different phenotypes of pigs regarding the differences in their susceptibility towards different variants of F4 strains [32]. Phenotype A pigs are susceptible to all three F4 variants, phenotype B only to F4ab and F4ac, while phenotype C pigs are susceptible to F4ab and F4ad. Phenotype D pigs are only susceptible to F4ad variant and phenotype F pigs to the F4ab variant. The affinity of phenotype E pigs has not been confirmed so far to any variant of F4 strain [33].

All F4 variant strains, especially F4ac adhere significantly more to jejunal than to duodenal villi [34]. This is because the majority of receptors are present in the mid-small intestine. In the

proximal and distal part of the small intestine, they are present in smaller amounts and entirely absent in the caecum and colon of susceptible pigs [35].

The most intriguing feature of F4 fimbrial intestinal receptors is their structure. Intestinal brush border receptors for F4 fimbriae are characterized as glycoconjugates [17]. Sellwood suggested that the receptors are either glycoproteins or glycolipids in nature, because β-D-galactose and periodate oxidation significantly interfere with their activity [36].

Furthermore, Gal β 1-3GalNAc and Fucα1-2Gal β 1-3/4GlcNAc oligosaccharide sequences are proposed to mediate the binding of receptors to F4ac fimbriae [37]. The F4ac variant of ETEC is the most widespread strain of ETEC that causes PWD [38].

2.1.2.2 F18 fimbriae

The F18 strain of ETEC also causes PWD worldwide. F18 fimbriae are long, flexible structures, characterized by a zigzag pattern. They mediate adherence to the intestinal epithelium in pigs older than 3 weeks of age, and not in younger pigs [14]. In the past, this strain was designated also as F107, 2134P or 8813, but now it is known that those are different names for two antigenically different variants of F18, namely F18ab and F18ac [5]. The F18ac variant is easily expressed *in vitro* and it is more commonly isolated from PWD cases than the F18ab variant, which is expressed poorly *in vitro* [14].

F18 positive ETEC strains regularly produce heat-stable enterotoxins STa and STb, but rarely heat-labile enterotoxin (LT) [39]. Structurally, F18 is comprised of a major subunit FedA, and a minor subunit, such as FedF that is responsible for adhesion [40]. The nature of the F18 receptor is unclear, however, it is suspected that H-2 histo-blood group antigens (HBGAs) and A-2

HBGAs may be involved in the binding of F18 positive *E. coli* to the host [41]. Some pigs are resistant to colonization by F18 positive ETEC due to the lack of receptors for F18 [12].

2.2 The prevention and control strategies for PWD

In the recent years, attempts to find the most effective strategy for prevention and control of PWD have intensified, attracting the interest of many researchers. The reason for that lies primarily in the fact that control of PWD by prophylactic antibiotic treatments done for years is not effective anymore due to the emergence of antimicrobial resistance [5]. Pressure to reduce the use of antibiotics in animal production and to explore alternative strategies is growing from year to year as the number of resistant bacteria in the intestine of animals increases [42]. The focus of this review will be on major preventive initiatives.

2.2.1 Chemoprophylaxis

During the course of PWD, pigs must be treated parenterally, because they eat or drink very little. Hence, antimicrobial substances have to be able to reach the intestinal lumen to control bacterial proliferation. The most commonly used antibiotics are amoxicillin/clavulanic acid, fluoroquinolones, cephalosporins, apramycin, ceftiofur, neomycin, or trimethoprim [15]. Due to the nature of the swine industry, there is the widespread practice of using these antimicrobial agents for preventive feed medication of affected herds [5]. This prophylactic approach to PWD resulted in the development of bacterial resistance to apramycin, neomycin, and trimethoprim or

trimethoprim-sulfamethoxazole [43]. Therefore, this approach for prevention and control of PWD has become unattractive.

2.2.2 Management

The major management measures for prevention and control of PWD are focused on hygiene and reduction of stress. In particular, the environment of the weaner units plays an important role in the epidemiology of PWD, as a major source of ETEC and stress for weaned piglets. Hence, general management measures are aimed at reducing any form of stress, such as transportation, chilling, mixing of litters, and introduction to new pens [15]. Another important factor is weaning age of piglets, which has been steadily decreasing in North American swine farms. This time has been reduced from previous practice of 5-6 weeks, to the current 3 weeks of age, or even less [44]. Particularly, early weaning represents a risk factor for development of PWD, because piglets do not develop strong humoral immunity for protection against ETEC infection before 12 weeks of age [45]. Routine cleaning and disinfection are usually insufficient to break the cycle of infection [46], but the transmission of PWD can be prevented by strict hygienic measures [15].

2.2.3 Breeding of resistant pigs

Not all pigs are susceptible to F4 and F18 strains of ETEC. Susceptibility and resistance of pigs to infection is closely associated with the ability of ETEC to colonize intestinal epithelium. Thus, in resistant pigs small numbers of ETEC bind to enterocytes and produce enterotoxins, however the amount is not sufficient to induce secretory diarrhea. This phenomenon can be

explained either by a small number of receptors present in the brush borders, or non-functional receptors that are not capable of binding bacteria [33]. Nevertheless, resistance of pigs to ETEC infection is inherited via an autosomal recessive gene [47]. This fact has stimulated researchers to consider breeding of resistant pigs as one of preventive strategies against PWD. Regardless of the current progress of this approach, there are certain disadvantages to it, such as risk of co-selection of unwanted traits closely linked with loci coding for the F4 and the F18 receptors, emergence of new adhesive variants of ETEC and lack of economical laboratory techniques for large-scale selection of resistant pigs. It is known that F4 strain resistant sows do not secrete F4 specific antibodies in colostrum, so heterozygous piglets may be at a higher risk of developing neonatal diarrhea [5].

2.2.4 Immunoprophylaxis

Immunoprophylaxis against PWD is achieved by passive and/or active immunotherapy. Insufficient passive immunity acquired from the sow is one of the main factors that contributes to PWD. There are several strategies that can be employed to improve it. Ingestion of anti-F4 or anti-F18 specific antibodies in weaned piglets has been achieved by adding dried porcine blood plasma [48] or chicken egg yolk [49] to post-weaning feed. These two approaches were implemented based on previous studies demonstrating that intestinal colonization with ETEC was inhibited in weaned pigs fed with supplementary porcine milk [50]. Although the feed additives based on dried porcine blood plasma demonstrated significant inhibitory effect on ETEC colonization with subsequent prevention of PWD, they showed as an expensive strategy for control of PWD. Furthermore, they were banned in Europe due to potential health risks, being sub-product of animal origin [5]. On the other hand, use of chicken yolk is a relatively

inexpensive strategy, because large quantities of specific IgY antibodies can easily be produced. When weaned pigs, fed with egg-yolk antibodies from chickens immunized with purified F4 are challenged with F4-positive ETEC, they are affected only by transient diarrhea and no mortality, in comparison to control piglets [49].

The active immunization of sows (e.g. *Escherichia coli* bacterin vaccine) is the most effective current strategy for control of neonatal diarrhea induced by ETEC. However, at the present time, there is no commercial vaccine that can fully protect weaned pigs against PWD.

There are two types of vaccination protocols against porcine colibacillosis. Maternal vaccination with a combination of fimbrial antigens and oral immunization of piglets with either live attenuated or live wild-type avirulent *E. coli* strains [51]. In the first protocol, active immunization of sows incites more systemic and less mucosal immunological protection in piglets, and appears to be not entirely effective against colonization of ETEC [52]. Furthermore, it can potentially suppress the mucosal immune response of subsequent oral immunization [53]. However, the presence of specific F4 antibodies in colostrum and milk during the suckling period has proven to protect piglets against ETEC intestinal colonization [50].

Some studies demonstrated that use of purified F4 fimbriae as the antigen (oral subunit vaccines) is more effective in stimulating the mucosal immune response [54] than attenuated *E. coli*. Ingested F4 fimbriae were demonstrated to have a strong immunogenicity resulting in production of antibody of IgM isotype in Peyer's patches, lamina propria, mediastinal lymph nodes, and blood. Administration of these vaccines in drinking water to weaned or unweaned piglets one week before the expected onset of diarrhea was demonstrated to prevent intestinal colonization of ETEC [54]. Accordingly, the F4 fimbriae and/or its major subunit FaeG continue to be exploited for development of new vaccines. For example, incorporation of the FaeG

subunit into feed through genetic modification of plants, such as alfalfa or transgenic tobacco, has been successful [55-56]. Joensuu et al., demonstrated that FaeG adhesins, synthesized in the tobacco plant and in alfalfa were stable in piglet gastric and intestinal *in vitro* conditions and could bind isolated piglet villi and inhibit subsequent F4 positive ETEC binding. However, immunization of piglets with recombinant FaeG adhesin induced a weak F4 specific humoral response [55-56]. On the other hand, the recombinant glycosylated FaeG expressed in barley endosperm was able to induce biologically active F4 specific antibodies in mice [57]. Based on these encouraging results it is anticipated that these research strategies may contribute to the future prevention of PWD in the modern swine industry.

2.2.5 Probiotics

Probiotics represent a large group of feed supplements containing live microorganisms, which positively affect the host intestinal health. There are several mechanisms believed to be responsible for the beneficial effects of probiotics on intestinal microflora [58]. The first mechanism involves the competition between probiotic and pathogenic microorganisms for attachment to intestinal epithelial receptors [59] resulting in reduced colonization of pathogens and prevention of the disease. The second mechanism is the potential effect of probiotics on nutrients from feed in the intestine, making them easily digestible and more available for absorption. In addition, probiotics synthesize antimicrobial substances and other inhibitors thus affecting growth of pathogens in the intestine [60]. Finally, it has been confirmed that they stimulate the local immune system resulting in increased secretion of immunoglobulins [61], and production of cytokines [62].

Use of the probiotics in prophylaxis of PWD currently attracts the great interest among many researchers. Many studies have explored the effect of probiotics on ETEC and PWD by testing various combinations of strains of bacteria, doses, and interactions with pharmaceuticals [5, 63-66]. It has been confirmed that use of *Rhizopus*-fermented or *Bacillus*-fermented soybeans (viable spores of *Bacillus licheniformis* and *Bacillus toyoi*) reduced the severity, duration and mortality of PWD in piglets [67]. Recently, there are reports that lactic acid producing bacteria, *Pediococcus acidilactici* and yeasts, *Saccharomyces cerevisiae boulardii* improve intestinal defences against F4 positive ETEC, by reducing ETEC F4 attachment to the intestine and by increasing production of proinflammatory cytokines involved in innate immunity against F4 positive ETEC. If used together, *P. acidilactici and S. cerevisiae boulardii* can increase significantly the expression of IL-6, IL-8 and TNF-α in the ileum of the pigs challenged with F4 *E. coli* [65]. It is confirmed that intestinal epithelial cells secrete these cytokines and that they play an important role in stimulation of local B-cells to produce IgA [68].

2.2.6 Prebiotics

Prebiotics are non-digestible food ingredients (e.g. non-starch polysaccharides, dextrins, inulin, lignin, waxes, pectins, beta-glucans, and oligosaccharides) able to stimulate the proliferation of probiotics in the gastrointestinal tract [66]. Their mechanism for stimulation of the growth of lactic acid and bifidobacteria (genus *Bifidobacterium*) is based on the ability to increase the volatile fatty acid, lactic acid and ammonia content in the digestive tract [69].

The most used and studied prebiotics to date are mannanoligosaccharides (MOS). They are isolated from the yeast cell wall and their protective effects are achieved by preventing the binding of pathogens to intestinal epithelial receptors [63]. In addition, MOS inhibit the growth

of different pathogens (*E. coli, Salmonella typhimurium, Clostridium botulinum and C. sporogenes*), stimulate the proliferation of probiotics (*Lactobacillus casei, L. acidophillus and L. delbrücke*) and incite a mucosal immune system response [58]. It has been confirmed that MOS have positive effects on the growth performance and overall health status of weaned pig [70].

There are reports about positive effects on human and animal health with other prebiotics such as fructooligosaccharides, galactooligosaccharides, and xylooligosaccharides [66, 71-73].

The prebiotic activity of fructooligosaccharides has been confirmed in animal and human trials [66]. The general mechanism of their action is by promoting growth of bifidobacteria in the human colon, which can result in excretion of natural antimicrobial activity against various Gram-positive and Gram-negative intestinal pathogens [66]. The combination of *Lactobacillus plantarum,* maltodextrin and fructooligosaccharides given orally to suckling piglets has proven to inhibit the adhesion of *E. coli* O8:K88 to the mucosa of the jejunum, ileum and colon [74].

Galactooligosaccharides and xylooligosaccharides are non-digestible and acid-stable carbohydrates [66]. They have shown the ability to significantly inhibit adhesion of *L. monocytogenes* to the intestinal epithelium *in vitro* [75] and decrease the numbers of intestinal *L. monocytogenes* in ileal samples from guinea pigs *in vivo* [76]. However, their potential use for prevention and control of PWD has never been reported and needs to be explored more in the future.

2.2.7 Non-antibiotic substances

There is a wide range of different non-antibiotic substances described in the literature and considered as potential alternatives in the prophylaxis of PWD.

Zinc oxide is a remarkably potent medication for prevention of PWD [5]. Its ability to efficiently reduce diarrhea and mortality as well as improve growth in pigs is related to many dietetic properties of zinc [5]. Zinc is a component of many enzymes responsible for amino-acid and protein metabolism [77]. Hence, it stabilizes intestinal mucosa, improves the diversity and functions of intestinal microflora, inhibits the growth of many pathogens and enhances the immune responses of the body against infections [78-81]. However, if high levels are used, there is a potential environmental risk for heavy metal contamination of the soil [5].

Certain organic acids in feed have antibacterial and antifungal properties, so they are commonly used in animal nutrition [58]. The antimicrobial effect of organic acids is associated with their ability to decrease feed pH, penetrate the cell wall and destroy microorganisms [82]. Growth rate and efficiency of feed conversion is significantly improved in weaned pigs by supplementing starter diets with a pure formic acid [83], or mixtures of organic acids (formic, citric, propionic and benzoic acids) [84]. In addition, piglets fed diets supplemented with organic acids (1.6 % lactic acid, 1.2% formic acid, 1.2% malic acid, 1.5% citric acid or 1.5% fumaric acid) had reduced incidence and severity of post-weaning diarrhea [85].

For centuries, various plants extracts have been used for treatment of human and animal diseases in traditional medicine. Recently, all of them are classified under the one group, natural feed additives (plants and spices), and they have started to attract considerable attention as alternatives to antibiotics [58]. Various antimicrobial, anti-inflammatory, anti-oxidative and anti-parasitic properties of these substances have been described in the literature [86-88].

Garlic, thyme, oregano, cloves, cinnamon, juniper extracts and plant essential oils are all considered as potentially very effective antimicrobials [58]. Bromelain, a protease from pineapple stems, if orally administered to pigs, can reduce the colonization by F4 positive ETEC

[89] and reduce the incidence of post-weaning diarrhea [90]. Nevertheless, a major disadvantage of plant extracts lies in the fact that they are unstable in feed formulations [91].

Use of natural and modified clay minerals in prophylaxis of enteric disease is an emerging research area. The natural clays (bentonite, zeolite, kaolin, etc.) possess high adsorption capacity, due to their stratified structure. Consequently, if added to a diet, they can bind and immobilize toxic materials and decrease their biological availability and toxicity in the gastrointestinal tract of animals [92]. Kaolin-based medications are already used in human medicine for the therapy of enteric diseases [93-94]. In the literature, there are reports that a Cu^{2+}-enriched clay mineral, such as montmorillonite that significantly decreased the incidence of post-weaning diarrhea in piglets [95].

The variety of strategies presented in this section indicates that several alternative approaches have been proposed and their effectiveness is variable for prevention of PWD. However, at this time, not a single strategy has been proven to be absolutely effective [5], which makes this disease even more attractive for some future research ventures.

2.3 Potential protective substances in porcine milk against PWD

Although the chemical and nutritional composition of porcine milk has been evaluated extensively [96-97], relatively little is known about its non-immunoglobulin protective properties against gastrointestinal diseases. There are a few studies reporting various potential substances in porcine milk that are able to inhibit the colonization and growth of various pathogens, including ETEC [11, 98-99].

As previously described for human milk [100], the following main potential antimicrobial components of porcine milk will be reviewed: antibodies, multifunctional agents, and homologues of host cell surface glycoconjugates.

2.3.1 Immunoglobulins

The immunoglobulin (Ig) content in porcine milk significantly changes throughout lactation. Colostrum has a high concentration of Ig (~125.9 g/L), which markedly declines during the first few days of lactation (~7.3 g/L). The major classes of Ig in porcine colostrum and milk are IgG, IgM and IgA. IgG concentration is highest in colostrum (~61.8 g/L) and starts to decrease as lactation progresses (after 42 days, ~0.8 g/L), while an increase as lactation progresses in the concentration of IgM is moderate (~1.4 g/L) and marked for IgA (~5.6 g/L) (40% of total milk protein at the end of lactation) [96].

Neonatal piglets are dependent on antibodies absorbed from colostrum right after the birth (within 24-36 hrs), because little or no placental transfer of antibodies occurs during gestation [101-102].

Humoral immunity against ETEC infection in pigs is delivered through sow's colostrum, antibodies in milk, and later in life through the intestinal mucosal immune response [15]. During a sow's pregnancy, intestinal lymphocytes stimulated by ETEC antigens, migrate to the mammary gland and produce specific IgA, IgM and IgG. Even though neonatal piglets in their first weeks of life begin to synthesize mucosal IgM and later IgA, this is not sufficient for immunological protection. Hence, colostrum and milk remain major sources of Ig for protection against ETEC infection [45]. If piglets fail to receive sow's colostrum and milk in early lactation, due to piglet deformities, sow's non-exposure to ETEC, agalactia, or generalized infection, they

will be highly susceptible to ETEC infections [15]. Weaned piglets fed with milk obtained from sows in late lactation were protected against colonization of ETEC and did not develop PWD [50]. This confirms that immunoglobulins and potentially other non-immunoglobulin substances present in porcine milk are most likely responsible for protection of piglets against ETEC infection.

2.3.2 Multifunctional agents

Multifunctional agents, sometimes termed bioactive substances, are considered an important part of the innate immunity of milk. These components provide neonates primarily with nutrients, but some of them, either in their native form or partially digested, can provide a broad range of protection for newborns against infection [103]. Lactoferrin, lysozyme, monoglycerides and fatty acids are the best known in the literature at this time.

2.3.2.1 Lactoferrin

Lactoferrin in porcine milk is an iron-binding glycoprotein with a molecular weight of 74.5 kDa. The concentration of lactoferrin at parturition ranges from 1.1-1.3 mg/ml, but the level decreases sharply during the first week of lactation [96]. The biological role of lactoferrin is not entirely clear, but the literature indicates that this multifunctional protein has broad antimicrobial and anti-inflammatory properties [104]. Bacteriostatic and bactericidal effects of lactoferrin are directed against a variety of microorganisms, including several strains of *Escherichia coli* [105-106]. The iron-free form of lactoferrin has a bacteriostatic effect, based on its ability to deprive the bacteria of unbound iron, making it unavailable for their metabolic activities *in vivo* [103].

The bactericidal activity of lactoferrin is still not definitively explained, but it is documented that lactoferrin causes a significant release of lipopolysaccharide (LPS), and damages the outer membrane of Gram-negative bacteria [107]. Also, it enhances the antibacterial activity of other multifunctional agents, such as lysozyme [108]. Pepsin digestion of lactoferrin produces lactoferricin B, a positively charged peptide of 18 amino acids with potent antibacterial activity for a wide spectrum of bacterial pathogens [103]. Oral administration of bovine lactoferrin to pigs demonstrated protection against endotoxic shock [109]. Porcine lactoferrin has affinity for LPS isolated from F4 positive *Escherichia coli* [99].

Another protective milk protein and part of innate immunity is lysozyme. In human milk the amount of lysozyme varies from 10 to 100 μg/L. Lysozyme damages cell walls of Gram-negative bacteria by a breaking critical linkage in the peptidoglycans of bacterial cell walls [103].

Triglycerides in milk provide mainly energy for newborns. However, when they are digested into fatty acids (linoleic and lauric acid) and monoglycerides, they also act as strong detergents for pathogen membranes [103].

2.3.3 Glycoconjugates

Glycoconjugates in milk consist of a wide group of molecules exclusively found in large amounts at early stages of lactation [110]. These components possess conjugated carbohydrate moieties, which often have a similar or identical structural pattern as host target cell receptors specific for pathogen adherence. Hence, they are sometimes referred to in the literature as homologues of cell surface glycoconjugates and a potentially significant component of innate immunity [111]. Moreover, some authors hypothesize that in newborns, glycoconjugates and

multifunctional agents form a primary line of defense against enteric pathogens during a period that occurs between the time of the mother's exposure to the pathogen and production of secretory antibodies against it in her milk [112]. Glycoproteins, glycolipids, and oligosaccharides play the largest role among them [100].

Several oligosaccharides and glycoproteins from human milk have been shown to block binding of specific pathogens to host cell receptors [112]. Particularly, the fucosylated oligosaccharide fraction of human milk demonstrated inhibition of adherence of *Streptococcus pneumoniae* to target cells *in vitro* [113]. In addition, it was confirmed that they can block the binding of a heat-stable toxin of enteropathogenic *Escherichia coli* to the extracellular portion of guanylate cyclase *in vitro*, which can be beneficial for prevention of secretory diarrhea in infants [114]. Fucosylated structures also inhibit binding of *Campylobacter jejuni* to host cell receptors and limit its colonization *in vivo* [100]. Binding of Norwalk virus to the duodenal enterocytes of infants can be blocked by α1,2-fucosyl glycans from human milk *in vitro* [112]. Human lactadherin inhibits binding of all major strains of human rotavirus to their target cells subsequently preventing diarrhea in infants [115]. Porcine lactadherin was confirmed to have binding affinity for F4ac fimbriae and the ability to decrease binding of F4ac positive *E. coli* to intestinal villi *ex vivo* [11]. Lactadherin is a major protein of Milk Fat Globule Membrane (MFGM) [10].

2.3.3.1 Milk fat globule membrane proteins

The lipids in the milk are secreted as droplets coated with a cellular membrane, called milk fat globule membrane (MFGM). These membranes consist of three layers that encase a central triglyceride core. The interior layer originates from the endoplasmic reticulum of lactating

mammary epithelium. The outer surface of milk fat globule is formed of two layers consisting of various proteins and polar lipids derived from apical membranes of the glandular epithelial cells [116]. The proteins associated with the milk fat globule membrane are called MFGM proteins. In this section, only major proteins identified in porcine milk will be reviewed [117].

Butyrophilin (BTN) is the most abundant protein of MFGM in many species, including pigs [10, 117]. Butyrophilin is an integral, type I membrane glycoprotein having a molecular weight of approximately 60 kDa [118]. The role of BTN in porcine milk is still not entirely explained, but in other species is well documented. It was hypothesized that a complex formed between BTN, xanthine dehydrogenase (XDH) and adipophilin (ADPH) in MFGM is primarily responsible for regulation of milk fat secretion [119-120]. However, recent studies suggest that BTN has an essential role in regulation of secretion of milk fat [121]. If the BTN gene is eliminated in mice, milk fat will lack MFGM, and will be highly unstable (extremely large lipid droplets), while the concentration of skim milk proteins will not be affected [122]. BTN belongs to the large immunoglobulin superfamily, which contains a many adhesive proteins with significant biological functions, such as receptors or inhibitors of T-cell activation [123-125]. There is a report that porcine BTN demonstrated significant binding capacity for F4ac fimbriae of ETEC during an *in vitro* study [117].

Milk fat globule-epidermal growth factor-factor 8 (MFG-E8), also known as lactadherin (LAD) in humans, or PAS 6/7 in bovine, was initially discovered as a major component of the MFGM of several species, but later research revealed its ubiquitous pattern of expression in different cells and tissues [126]. Paradoxically, while functions of lactadherin in milk still remain undefined, research in other expression sites is thriving. The most remarkable function of LAD discovered up to now is its ability to promote the clearance of apoptotic cells by forming bonds

between phagocytes and apoptotic cells [119, 127]. LAD also promotes cellular adhesion, through interaction between its N-terminal epidermal growth factor (EGF)-like domain with RGD cell adhesion sequence and cell integrins [128]. LAD exhibits a proangiogenic effect (neovascularization) in tissue regeneration, because of its integrin-mediated signaling of vascular endothelial growth factor [129]. Human LAD possesses inhibition activity against all major strains of human rotavirus, a significant cause of diarrhea in infants, both *in vitro and in vivo* [115, 130]. LAD from porcine MFGM demonstrated high affinity for F4ac fimbriae, the potential to prevent binding of F4ac positive *E. coli* to intestinal villi *ex vivo* [11] and a strong interaction with F4ac fimbriae *in vitro* [117].

Xanthine dehydrogenase/oxidase (XDH/XO) is a widely distributed protein in a variety of mammalian tissues. This complex cytosolic enzyme has been known for a long time as one of the major constituents of bovine MFGM, located along its inner layer. XDH/XO consists of two identical subunits of 147 kDa [131]. Each subunit contains one molecule of molybdenum, one of flavin adenine dinucleotide, and two iron-sulfur redox centers [132]. XDH/XO has a crucial role in purine catabolism, catalyzing the oxidation of hypoxanthine to xanthine and the oxidation of xanthine to uric acid [10]. Protein–protein interactions are documented to be responsible for the formation of a complex among XDH/XO, BTN, and ADPH, and regulation of secretion of milk fat. An experiment with knockout mice has revealed that XDH/XO and BTN are essential for milk fat secretion [119]. XDH/XO has two interconvertible forms, XDH, which predominates *in vivo*, and xanthine oxidase (XO). Both forms of the enzyme reduce molecular oxygen, and generate the reactive oxygen species (ROS), superoxide anion, and hydrogen peroxide. Therefore, XDH/XO has been described as a destructive agent in ischemia-reperfusion injuries, but moreover as a highly potent antimicrobial agent [131].

Perilipin 2, ADPH, or adipocyte differentiation-related protein, are all names for one protein implicated in the storage and metabolism of lipids in many species. In bovine MFGM, this protein is often overlooked, because of a similar molecular weight as LAD (46 kDa) [10]. Nevertheless, two-dimensional (2D) SDS-PAGE reveals their different isoelectric points [10]. ADPH can be generated in a variety of cells and tissues, not exclusively in adipocytes, but also in lactating mammary epithelial cells, Sertoli and Leydig cells in the testis, adrenal cortical cells, and others [133]. However, knowledge about physiological functions of ADPH is currently inadequate. The complex that forms between ADPH, BTN, and XDH/XO is described as related to regulation of secretion of fat in milk [119]. ADPH has a role in lipid biogenesis and cellular uptake of long-chain fatty acids [134-135]. In a recent study, ADPH exhibited strong binding ability for F4ac fimbriae of ETEC *in vitro* [117].

Fatty acid binding protein (FABP), approximately 13 kDa, is a component of bovine MFGM [10]. Originally, FABP was identified as a growth inhibitor of mammary carcinoma cells, and called mammary-derived growth inhibitor [136]. FABP in MFGM is a member of the intracellular FABP family and is most similar to heart FABP [137]. Several roles of FABP are proposed, and all are associated with lipid metabolism [10]. FABP inhibits growth and stimulates differentiation of mammary gland epithelium [10]. Recently, it was demonstrated that FABP from porcine milk interacted with F4ac fimbriae of ETEC *in vitro* [11, 117].

Acyl-CoA synthetase 3 (ACS3), also known as long-chain-fatty-acid-CoA ligase 3, and sometimes referred to inaccurately as Acyl-CoA carboxylase, is a minor protein of MFGM [138]. Functionally, it is regulatory enzyme, essential for *de novo* lipid synthesis, fatty acid catabolism, and remodeling of membranes [139]. Based on the length of the carbon chain of the fatty acids specific for the different acyl-CoA synthetases, five sub-families of ACS have been

characterized at this time. ACS belongs to the large long-chain fatty-acid-coenzyme A ligase family that activates the degradation of complex fatty acids [139]. It has been shown that several acyl-CoA synthetases, together with some other substances related to lipid transport synthesis and secretion, were upregulated up to 5 times in bovine MFGM obtained at the 7th day of lactation in comparison to colostrum MFGM [120]. Although ACS3 is present in porcine MFGM in small amounts compared to the other major proteins, it has showed a strong binding ability for F4ac fimbriae of ETEC *in vitro* [117]. In the future, more studies will be required to resolve all physiological roles of ACS of MFGM.

Initially, Atroshi et al., reported that porcine MFGM can act as a target for binding of F4 positive *E. coli* [8]. Furthermore, it was demonstrated that porcine milk MFGM have the potential to inhibit the binding of F4 fimbriae to porcine intestinal brush borders [9]. Recent studies revealed that certain proteins of porcine MFGM, such as lactadherin and fatty acid binding protein have binding affinity for F4ac fimbriae of ETEC [11], and that lactadherin, butyrophilin, adipophilin, and acyl-CoA synthetase 3 interact with F4ac-fimbriae *in vitro* as demonstrated by overlay Western blot technique [117].

3 RESEARCH HYPOTHESES AND OBJECTIVES

3.1 Rationale

F4ac positive enterotoxigenic *Escherichia coli* (ETEC) is one of the most common pathogens that can cause neonatal and post-weaning diarrhea in piglets. The attachment of ETEC to the intestinal brush border is an essential step in the pathogenesis. It is always mediated by the same filamentous surface appendages called F4 fimbriae. The major structural subunit of F4ac fimbriae is composed of a protein possessing adhesive properties. However, sow milk contains many substances that can bind to F4ac preventing attachment of ETEC to enterocytes. These substances range from antibodies specific for F4ac to non-immunoglobulin substances such as lactadherin. The lactadherin was primarily isolated as an F4ac-binding protein from porcine skim milk. Since this protein is one of the structural proteins of Milk Fat Globule Membrane (MFGM), there was a reasonable interest in F4-binding affinity of the other MFGM proteins, which was initially investigated by overlay Western blot technique [117] and subsequently confirmed by affinity chromatography in this thesis.

3.2 Hypothesis 1

In addition to lactadherin [11], there are other proteins of the MFGM, which interact with F4ac fimbriae of *E. coli* in their native configuration (i.e. with preserved quaternary, tertiary and secondary structures).

3.3 Objective 1

To isolate and characterize F4ac-binding proteins of porcine MFGM by affinity chromatography.

3.4 Hypothesis 2

The F4ac–binding proteins isolated by affinity chromatography (objective 1) inhibit the attachment of enterotoxigenic *E. coli* to porcine enterocytes *in vitro*.

3.5 Objective 2

To evaluate the inhibitory effect of individual F4ac-binding MFGM proteins against attachment of F4ac-positive *E. coli* to porcine enterocytes *in vitro* by competitive ELISA.

4 IDENTIFICATION OF F4AC-BINDING PROTEINS OF PORCINE MILK FAT GLOBULE MEMBRANE

The affinity chromatography results described in this chapter were merged with previous results generated in our lab (Chapter 3 of Dr. Huang's MSc Thesis) and submitted for publication as the following manuscript:

P. Novakovic [*], Y.Y. Huang [*], B. Lockerbie, F. Shahriar, J. Kelly, D.J. Olson, J.R. Gordon, D.M. Middleton, M.E. Loewen, B.A. Kidney, E. Simko. Identification of F4ac-binding proteins of porcine milk fat globule membrane. Submitted to Developmental and Comparative Immunology. (* equal contribution)

4.1 Introduction

Enterotoxigenic *Escherichia coli* (ETEC) expressing the F4 is the most common bacterial pathogen that causes neonatal and post-weaning diarrhea (PWD) in piglets. At the present time, the F4ac variant of ETEC is the most dominant type worldwide [5]. An essential step in the pathogenesis of PWD is the attachment of ETEC to the intestinal brush border of weaned piglets. This attachment is mediated by bacterial filamentous surface appendages called F4 fimbriae. This mechanism of attachment enables the bacteria to resist peristalsis of the small intestine, in order to effectively colonize enterocytes and deliver enterotoxins [5]. The major subunit of F4 fimbriae is a protein in nature with adhesive properties for carbohydrates. The porcine receptors for F4 fimbriae are glycoproteins and glycolipids found both on the intestinal brush border and within mucus [17, 140]. Binding between F4 fimbriae and specific intestinal receptors has

characteristics of lectin-carbohydrate interactions, in which the fimbriae bind to glycan moieties of glycoproteins [141].

Sow milk contains substances that can bind to F4 fimbriae and potentially prevent colonization of ETEC to the intestine and subsequent development of PWD [50]. These substances range from antibodies specific against fimbrial antigens to non-immunoglobulin glycoproteins, such as lactadherin and fatty acid binding protein (FABP) [11]. Both, lactadherin and FABP originate from the structure that surrounds milk fat droplets, called milk fat globule membrane (MFGM) [10]. Previous studies reported that porcine MFGM can act as a target for binding of F4 positive *E. coli* [8]. In addition, it was demonstrated that porcine milk MFGM can inhibit the binding of F4 fimbriae to porcine intestinal brush borders [9]. A recent study using an overlay Western blot technique revealed that several porcine MFGM proteins interacted with F4ac fimbriae *in vitro* [117]. In this particular study MFGM proteins were separated by two-dimensional electrophoresis (2D SDS PAGE); therefore their tertiary and secondary structures were destroyed by complex chemical treatments of samples subjected to 2D SDS PAGE. Accordingly, the purpose of this study was to investigate F4ac-fimbrial binding affinity of MFGM proteins not exposed to harsh chemical treatment using affinity chromatography.

4.2 Material and methods

4.2.1 Bacteria

F4ac positive *Escherichia coli* (O149:K91:K88ac), isolated from a clinical outbreak of neonatal diarrhea of piglets, was kindly provided by Dr. Musangu Ngeleka, Prairie Diagnostic

Services Inc., University of Saskatchewan. Bacterial stocks in trypticase soy broth (TSB) (Becton Dickinson and Co, Sparks, MD) and glycerol (2:1) were prepared [142] and stored at –70°C.

4.2.2 Isolation of F4ac fimbriae

F4ac fimbriae were isolated and purified according to a previously published method [143], the only modification being the introduction of filtration of solubilized F4ac fimbriae through 0.20µm pore size filters (Nylon Membrane 0.2µm 47 mm PK/100, Millipore Corporation, MA, USA) before each step of precipitation at pH 4 during the purification process to prevent aggregation. Purified F4ac fimbriae were stored at -20°C in PBS (1.6 mM NaH_2PO_4, 9.4 mM Na_2HPO_4, 154 mM NaCl, pH=7.2). The fimbrial concentration was determined with a Quick Star Bradford protein assay (Bio-Rad Laboratories, Mississauga, ON) using a Spectra Max 340 PC Spectrophotometer with SoftMax Pro software (Molecular Devices Corporation, Sunnyvale, CA, USA). To determine purity of isolated F4-fimbriae, they were subjected to sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and visualized with Coomassie stain.

4.2.3 One-dimensional SDS-PAGE

SDS-PAGE (12%, discontinuous) electrophoresis (PAGE) was performed under reduced conditions using a glycine buffer system according to the instructions of the PAGE equipment manufacturer (Mini-PROTEAN® 3 Cell and PROTEAN® II xi Cell, Bio-Rad Laboratories, Mississauga, ON).

4.2.4 Western blot of F4ac

The isolated F4ac fimbriae were confirmed by Western Blot using Mini Trans-Blot Electrophoretic Transfer cell (Bio-Rad Laboratories, Mississauga, Ont., Canada). Briefly, the sample of isolated F4ac fimbriae was subjected to 1D SDS PAGE, transferred onto PVDF membrane (Bio-Rad Laboratories, Mississauga, Ont., Canada) using transfer buffer (25mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) under 100 V for 1 hour. The membrane was blocked in PBS containing 0.5 % Tween 20 (PBS-T) and 5% BSA for 1 h on shaker. Then the membrane was washed in PBS-T and incubated for 1.5 h with a 1:2000 dilution of rabbit anti-F4ac polyclonal antibodies (Dr J.M. Fairbrother, Faculte de Medecine Veterinaire, Universite de Montreal, Saint-Hyacinthe, QC, Canada). After four washes for 5 min each with PBS-T, the membrane was incubated with a 1:10,000 dilution of ECL Plex CyDye conjugated secondary antibody (GE Healthcare Bio-Sciences, Uppsala, Sweden) for 1 hour on shaker while protected from light. The membrane was then washed four times for 5 min in PBS-T, protected from light, and rinsed three times in PBS. Detection of the secondary antibody signal was done by scanning the membrane with a fluorescent scanner (Typhoon Trio, GE Healthcare Bio-Sciences, Uppsala, Sweden). Finally, the anti-rabbit Cy5 signals were measured at 633 nm (670-nm band-pass filter, i.e. 670 BP 30) with photomultiplier's (PMT) voltage of 500 V.

4.2.5 Isolation of MFGM proteins

MFGM proteins were isolated from porcine milk fat according to previously published methods with minor changes [144-145]. Porcine milk was obtained from healthy sows 21 days after farrowing (Prairie Swine Centre Inc., Saskatoon, SK, Canada) following subcutaneous

administration of 2 ml of oxytocin (MTC Pharmaceuticals, Cambridge, ON, Canada). The milk was expressed manually and kept at 4°C during transportation to the laboratory. The milk fat was obtained by centrifugation at 3600 x *g* for 30 min at 4°C. The skim milk was removed and the fat fraction was washed three times with PBS, followed by centrifugation at 1500 x *g* for 20 min at 20ºC. The supernatant creamy layer was collected, resuspended in PBS, and subjected to intensive sonication (6 x 60 sec with cooling intervals on ice) to break and release MFGM proteins which were harvested by ultracentrifugation at 100,000 x *g* for 90 min at 4ºC. The pellet was resuspended in PBS and sonicated for 30 sec in order to prevent formation of protein aggregates. Concentration of proteins was measured by Quick Star Bradford protein assay and sample was filtered through 0.45 mm filters (Whatman 25mm GD/X Syringe filter, Clifton, New Jersey, USA) prior to affinity chromatography.

4.2.6 Preparation of affinity matrix

Approximately 50 mg of the purified F4ac fimbriae were coupled to 3 g of CnBr-activated Sepharose 4B (GE Healthcare Bio-Sciences, Uppsala, Sweden) according to the manufacturer's instructions. A negative control column, with the omission of F4ac fimbriae, was similarly generated.

4.2.7 Immuno-dot-blot

An immuno-dot-blot technique was used to confirm the efficiency of coupling of F4ac fimbriae to CnBr-activated Sepharose 4B, according to a previously published method [11]. Briefly, 100 µl of coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) containing 50 µl of

CnBr-activated Sepharose 4B matrix (GE Healthcare Bio-Sciences, Uppsala, Sweden) with or without coupled F4ac fimbriae were loaded into wells of Bio-Dot SF microfiltration apparatus (Bio-Rad Laboratories). 100 µl of PBS containing 10µg purified F4ac fimbriae were loaded as positive control. The samples were filtered through PVDF membranes by negative pressure, and then the membranes were subjected to the same Western blot technique that was used for confirmation of the isolated F4ac fimbriae.

4.2.8 Affinity Chromatography

Both the F4ac-CnBr-activated Sepharose 4B and negative control CnBr-activated Sepharose 4B matrices were packed into chromatography columns (XK 16/20, GE Healthcare Bio-Sciences, Uppsala, Sweden) which were then used to isolate F4ac-binding proteins from MFGM proteins by affinity chromatography using ÄKTA purifier 10 chromatography system (GE Healthcare Bio-Sciences, Uppsala, Sweden) controlled by Unicorn 5.11 software. The F4ac-CnBr-activated Sepharose 4B column was equilibrated with 5 column volumes of TBS buffer (10 mM Tris, 150 mM NaCl, pH 7.2) and then approximately 35 mg of MFGM proteins diluted in 24 ml of PBS was loaded to the column, which was then washed with 10 column volumes of TBS. The first (low affinity) elution was performed with 5 column volumes of 1 M NaCl in 10 mM Tris (pH 7.2) followed by 2 column volume of TBS, while the second (high affinity) elution was done using 5 column volumes of 0.1 M Glycine–HCl (pH 2.5). These two elution fractions were collected and pooled separately, dialyzed for 48 h at 4^0C against deionized H_20, using 1-kDa-cut-off cellulose dialysis membranes (Spectra/Por, Spectrum Laboratories, Inc., CA, USA), and then lyophilized in Freeze Dry System/Freezone 2.5 (Labconco Corp., MO, USA). The same procedure was repeated for the negative control CnBr-activated Sepharose 4B column. The

elution fractions were separated in 12% polyacrylamide gel using 1 D SDS-PAGE, visualized by Coomassie stain and identified by nanoLC-MS/MS.

4.2.9 NanoLC-MS/MS identification of proteins

F4ac-binding proteins isolated by affinity chromatography were submitted for nanoLC-MS/MS identification to the Institute for Biological Sciences, National Research Council, Ottawa, ON.

Briefly, proteins were excised from Coomassie stained SDS-PAGE, placed in clean Eppendorf tubes and destained overnight in 30% acetonitrile in 100 mM ammonium bicarbonate. The destained gel bands were reduced with DTT, alkylated with iodoacetamide and digestion overnight with sequencing grade trypsin (Promega, Madison, WI, USA) using standard laboratory protocols. The resulting digests were analyzed by nanoLC-MS/MS with data dependent acquisition using a NanoAquity UPLC system (Waters, Milford, MA, USA) coupled to a LTQ XL linear ion trap mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA). The samples were injected onto a 300 µm i.d. × 5 mm C18 PepMap100^{TM} trap (Thermo Fisher Scientific - Dionex, Sunnyvale, CA, USA), and separated on a 10 cm x 100 µm i.d. C18 column (Waters, 1.7 µm BEH130C18) at ~400 nL/minute, using a 30 minute gradient: 1-45% solution B (100% ACN/0.1% formic acid) in 19 minutes, 45-85% B over 2 minutes, 85-1% B in 1 minute, hold at 1% for 8 minutes. The peptide MS/MS spectra were searched against the NCBInr mammalian database using the MASCOT^{TM} search engine (Matrix Science, UK) for protein identification. The search parameters were: full tryptic cleavage, ≤1 missed cleavage, ≤1.5 Da for precursor ion, ≤1.2 Da for the fragment ions, oxidation (Met) and carbamidomethyl (Cys) as

variable modifications. For proteins with significance scores of 60 or less, all peptide MS/MS spectra were verified manually.

4.3 Results

F4ac fimbriae were isolated from bacterial culture, subjected to SDS PAGE, visualized by Coomassie stain and confirmed by Western blot (Figure 4-1). Purified F4ac fimbriae were coupled to CnBr-Sepharose affinity matrix (Figure 4-2) which was used for preparation of the affinity columns for the isolation of F4ac-binding proteins from porcine MFGM proteins. Affinity chromatography was performed with CnBr-activated Sepharose 4B (Figure 4-3) as negative control column and F4ac-fimbriae-CnBr-activated Sepharose 4B column (Figure 4-4) as experimental column. Elution fractions obtained in affinity chromatography were separated by 1D SDS-PAGE and stained with Coomassie blue G-250 (Figure 4-5). MFGM proteins bound to F4ac-fimbriae coupled to activated Sepharose (positive column) were eluted by low (1M NaCl) and high (0.1M Glycine-HCl pH 2.5) affinity elutions, separated by 1D SDS-PAGE, visualized by Coomassie stain and identified by nanoLC-MS/MS as xanthine dehydrogenase, acyl-CoA synthetase 3, lactotransferrin, butyrophilin, adipophilin, lactadherin, and fatty acid binding protein (Table 4.1). The same procedure was repeated with the negative affinity column (containing only activated Sepharose), but the elutions contained only trace amounts of proteins, and they were not taken for further analysis.

4.4 Discussion

Xanthine dehydrogenase, acyl-CoA synthetase 3, butyrophilin, adipophilin, lactadherin, and fatty acid binding protein were demonstrated to have binding affinity for purified F4ac fimbriae by affinity chromatography. These results are consistent with the previous study that investigated MFGM proteins interacting with F4ac fimbriae using overlay Western blot [11, 117].

The experiment with an affinity chromatography technique revealed very similar results to those generated by overlay Western blot. More specifically, acyl-CoA synthetase 3, butyrophilin, adipophilin, lactadherin, and fatty acid binding protein were confirmed to bind to F4ac fimbriae by both techniques. However, the experimental conditions used in affinity chromatography and overlay Western blot experiments differed significantly. In the overlay Western blot technique MFGM proteins were subjected to reducing SDS-PAGE treatment that included effects of a reducing agent, i.e. dithiothreitol (DTT) and a denaturing detergent, i.e. sodium dodecyl sulphate (SDS) and a high concentration of urea. It is known that SDS denatures native proteins from their tertiary and secondary protein structure to their primary structure, i.e. individual polypeptides. Furthermore, DTT reduces all disulfide linkages, which destroys tertiary protein folding and breaks quaternary protein structure, i.e. oligomeric subunits [146]. On the other hand, experimental conditions of affinity chromatography should not have affected secondary, tertiary or quaternary structures of MFGM proteins; therefore, it is assumed that they were preserved at the moment of interaction with F4ac fimbriae. The similar results generated by two different experiments (i.e. affinity chromatography and overlay Western blot) suggest these two possible explanations. First is that primary structures of MFGM proteins are mainly responsible for interaction with F4ac fimbriae. Another possibility is that binding affinity originates from a mechanism based on lectin-carbohydrate interaction. Evidence that such a possibility might be

correct is the example of human lactadherin, which demonstrated ability to inhibit binding of human rotavirus to target cells and prevent consequent diarrhea in infants. Removal of the terminal sialic acid from the carbohydrate moiety of this glycoprotein eliminated its inhibitory ability, which suggested that glycan residues were responsible for binding to rotavirus [115]. In addition to that, it was demonstrated that porcine lactadherin exposed to periodate oxidation treatment abolished its ability to decrease binding of F4ac positive *E. coli* to intestinal villi *ex vivo* [11]. The periodate oxidation was used to test if interaction between lactadherin and F4ac fimbriae was mediated by a carbohydrate-lectin reaction. However, if this kind of procedure is not optimized, it could potentially alter not just the structure of carbohydrates, but also the protein portion of glycoproteins, and introduce potential false results. For example, partial or complete inactivation of the antibody can occur under the periodate oxidation, particularly if the easily oxidizable amino acids, such as cysteine, cystine, methionine, tryptophan, are present in the antigen binding site [147]. However, if glycan moieties are responsible for affinity of MFGM proteins for F4ac, their potential identification and characterization may be used for the purposes of development of a new strategy for the prevention of PWD. At the present time, only data for posttranslational modification of bovine lactadherin are available in the literature. Bovine lactadherin contains both N- and O- linked glycans. Compositional analysis and mass spectrometry suggested that N-linked glycans comprise of two oligosaccharide structures, e.g. typical high-mannose containing sequence (Manα1→6 (Manα1→3) Manβ1→4GlcNAcβ1-→4GlcNAc) and GalNAcβ1→4GlcNAc [10]. The O-linked glycan of bovine lactadherin appeared to have a similar composition. However, study with lectins suggested that O- and N-linked glycans associated with bovine lactadherin changes significantly their glycosylation profile during early lactation, which complicates comparison between individual samples [10].

Interestingly, data from monosaccharide blocking studies indicated that terminal *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc) and galactose (Gal) may play a central role in the interaction of the F4 adhesin with brush border receptors [148]. On the other hand, glycosylation modifications of porcine LAD and other MFGM proteins remain entirely unknown. We suggest that future efforts have to be directed to the identification and characterization of porcine LAD glycan portions. Bovine BTN has three potential N-linked glycosylation sites containing Galβ1→4GlcNAc, GalNAcβ1→4GlcNAc and high-mannose (Manα1→2Manα1→3-Manα1→6 and Galβ1→4GlcNAcβ1→2Manα1→3) structures. BTN is not known to contain O-linked glycans [10]. It is not confirmed that bovine ADPH, FABP and acyl-CoA synthetase 3 are glycosylated, but posttranslational modifications in these proteins have not been extensively studied so far [10]. This also applies to the same proteins from porcine MFGM, and it should be taken into consideration as an objective of future research investigations. Particularly, since all these proteins demonstrated interaction with F4ac fimbriae, the experimental approaches that aim to determine the mechanism of this binding, should be prioritized.

Xanthine dehydrogenase was eluted by both low and high affinity elution buffers from the column containing F4ac-fimbriae coupled to CnBr-activated Sepharose 4B, but no interaction with F4ac was demonstrated with overlay Western blot. The reason for this discrepancy was not determined; however, the following is the most likely explanation. A protein complex formed by disulfide bonds among butyrophilin, adipophilin and xanthine dehydrogenase was described previously in MFGM [10]. Accordingly, it is possible that the entire complex was bound to F4ac fimbriae coupled to CnBr-activated Sepharose 4B, and subsequently eluted from it. Following reduced 1D SDS PAGE separation of the eluted proteins, xanthine dehydrogenase was also

present among F4ac-binding proteins. Since out of this protein complex only butyrophilin and adipophilin interacted with F4ac fimbriae in overlay Western blot, we suspect that the identification by affinity chromatography of xanthine dehydrogenase as an F4ac-binding protein was a false positive result.

On the other hand the identification of lactotransferrin, or lactoferrin, in low pH eluate was an unexpected finding. This soluble protein is commonly found in secretory fluids, such as milk, saliva, tears, etc. [149]. In our opinion, there are two possible explanations for this finding. First is that we are dealing with a completely different protein from the one present in fluids, i.e. membrane bound lactoferrin, described so far only in human MFGM [150-151]. The second explanation is possible contamination of isolated MFGM proteins with skim milk proteins, i.e. lactoferrin, which may occur particularly during the washing of milk fat in the isolation procedure. However, the absence of other more abundant F4ac-binding proteins of skim milk (i.e. β casein) in both affinity chromatography eluates indicates that contamination is unlikely. Nevertheless, F4ac-binding ability of lactoferrin has never been demonstrated before and requires more clarification in the future.

In conclusion, butyrophilin, adipophilin, lactadherin, acyl-CoA synthetase 3 and fatty acid binding protein 3 in porcine MFGM can bind to F4ac fimbriae *in vitro*. It is possible that these proteins serve as receptor analogues for F4ac fimbriae and may prevent attachment of *E. coli* to intestinal epithelium.

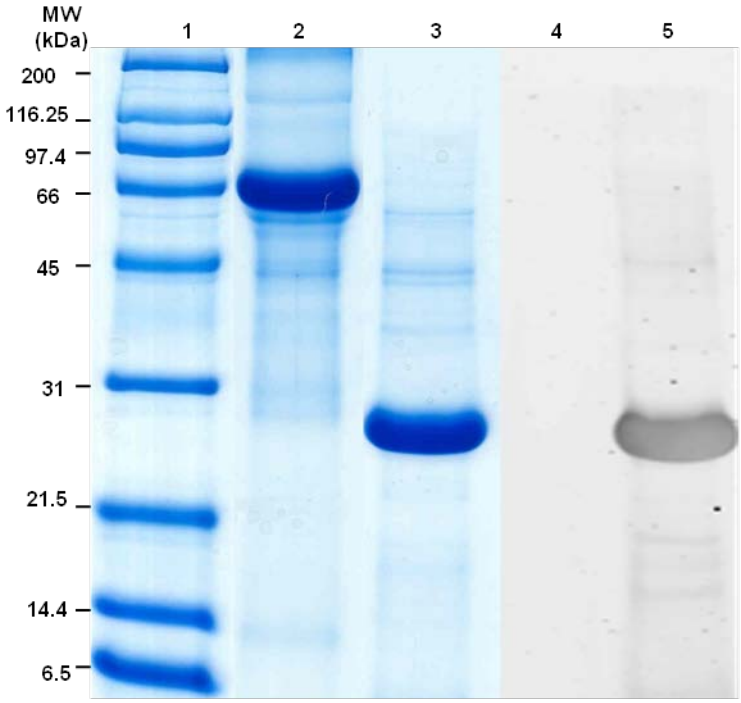

Figure 4-1 Identification of F4ac fimbriae. F4ac fimbriae isolated from enterotoxigenic *E. coli*, subjected to SDS-PAGE, visualized by Coomassie stain and detected by Western blotting using primary rabbit anti-F4-polyclonal antibodies and secondary anti-rabbit Cy5 antibodies (Lane 1 - Molecular Weight Markers; Lane 2 and 4 - Bovine serum albumin as negative control; Lane 3 and 5 - isolated F4ac-fimbriae)

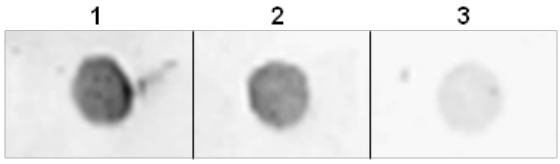

Figure 4-2 Immuno-dot-blot. 1) Isolated and purified F4ac fimbriae as positive control, 2) F4ac fimbriae coupled to CnBr-activated Sepharose 4B affinity matrix, and 3) CnBr-activated Sepharose 4B without F4ac fimbriae used as negative control affinity matrix tested with primary rabbit anti-F4-polyclonal antibodies and secondary anti-rabbit Cy5 antibodies

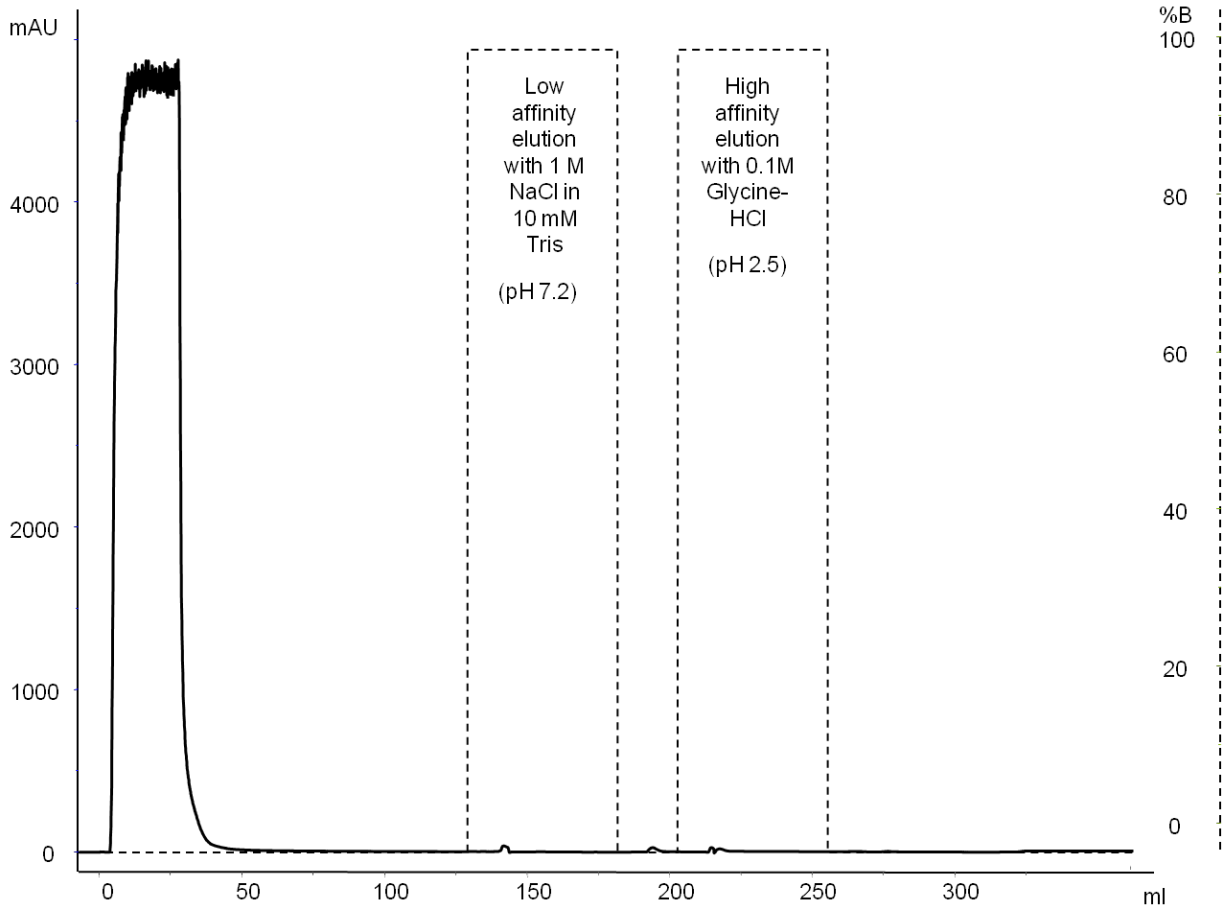

Figure 4-3 Affinity Chromatography with negative control column (CnBr-activated Sepharose 4B). 35 mg of MFGM proteins in 24 ml of PBS were applied to the column, washed for 10 column volumes and eluted with two different elution buffers. There were only minor elution peaks during both elutions, which were considered to be non-specific presumably due to changes in the buffers as observed also at the same time lag during the wash between two elutions.

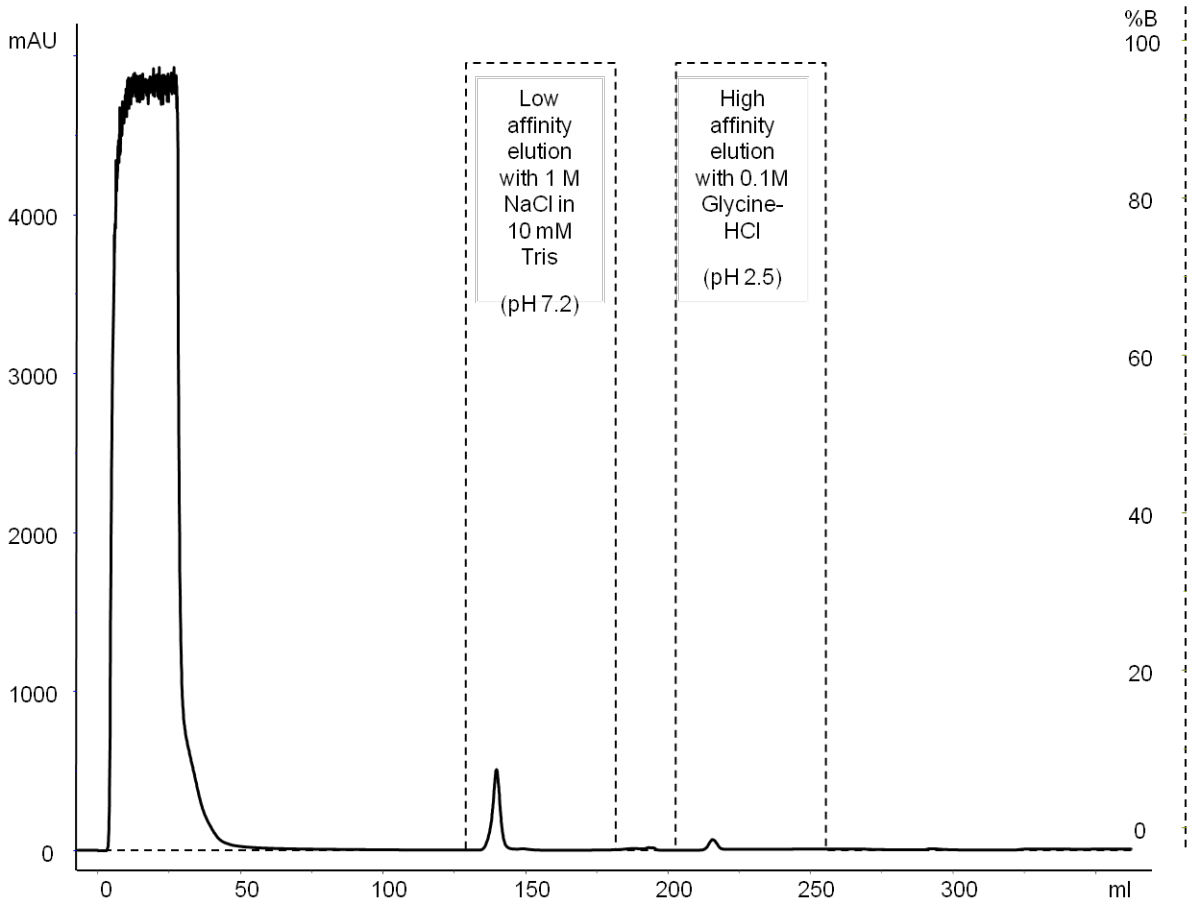

Figure 4-4 Affinity Chromatography isolation of F4ac-binding proteins from MFGM proteins using a column containing F4ac fimbriae covalently bound to CnBr-activated Sepharose 4B. 35 mg of MFGM proteins in 24 ml of PBS were applied to the column, washed for 10 column volumes and eluted with two different elution buffers. Prominent elution peaks were observed in both elutions.

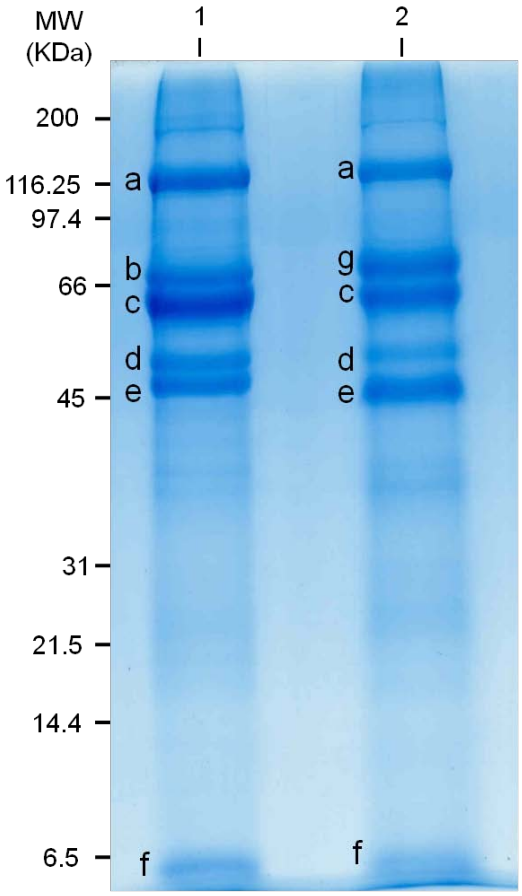

Figure 4-5 MFGM proteins eluted from positive affinity column (containing F4ac-fimbriae coupled to activated Sepharose), separated by one dimensional SDS-PAGE, stained by Coomassie Blue G-250 stain. Lane 1 – Low affinity elution with 1 M NaCl ; Lane 2 – High affinity elution (low pH 2.5); MFGM proteins identified by nanoLC-MS/MS: a) xanthine dehydrogenase, b) acyl-CoA synthetase, c) butyrophilin, d) adipophilin, e) lactadherin, f) fatty acid binding protein and g) lactotransferrin

Table 4.1 NanoLC-MS/MS identification of F4ac-binding MFGM proteins isolated by low (1M NaCl) and high (pH 2.5) affinity elutions.

Protein IDs	Mass (Da)[1]	Low affinity elution (1M NaCl) Score[2]	Low affinity elution (1M NaCl) Unique Peptides[3]	High affinity elution (pH 2.5) Score[2]	High affinity elution (pH 2.5) Unique Peptides[3]
Xanthine dehydrogenase	146706	609	24	459	18
Acyl-CoA synthetase 3	80245	1235	33	694	2
Lactotransferrin	77562	------	------	1554	31
Butyrophilin subfamily 1 member A1-like	59100	1038	26	908	25
Adipophilin	50180	1951	33	2310	39
Lactadherin	47819	956	21	830	22
Fatty acid binding	13219	415	8	370	5

1 - Calculated molecular weight of protein
2 - The protein identification score obtained by the MASCOT™ (Matrix Science, UK) search in NCBInr mammalian database (significance threshold $p<0.05$)
3 - Number of matched peptides

The previous study in our research laboratory [11, 117] demonstrated that several porcine MFGM proteins interacted with F4ac fimbriae *in vitro* and these findings were further confirmed by affinity chromatography (Chapter 4). Moreover, lactadherin, a major MFGM protein, was reported to inhibit the F4ac positive ETEC attachment to porcine intestinal villi *ex vivo* [11]. Accordingly, we hypothesized that the other F4ac-fimbrial binding proteins isolated from porcine MFGM inhibit the attachment of ETEC to enterocytes. However, testing of this hypothesis proved to be very challenging in the beginning due to the lack of effective techniques for purification of selected proteins from MFGM. The phase separation technique with Triton X-114 and size exclusion chromatography did not generate desired results. Finally, electro-elution was employed and individual proteins were isolated. To test the inhibitory effects of isolated MFGM proteins against ETEC attachment, competitive ELISA with an intestinal cell line or primary enterocytes was developed and used instead of a bacterial attachment assay using intestinal villi *ex vivo*.

Prior to these experiments, attempts were made to determine the strength of binding of individual MFGM proteins to F4ac fimbriae by Surface Plasmon Resonance (SPR) technique. SPR is an optical detection technique, which detects refractive index changes in close proximity to a planar metal (gold) surface. When the analytes (e.g. MFGM protein) bind to ligand molecules (e.g. F4ac fimbriae) immobilized on the surface, a change in the interfacial refractive index is detected and quantified by the instrument as a SPR signal, expressed in resonance units, RU (RU is equivalent to one picogram per square millimetre of sensor surface) [152]. Those proteins that would show a strong signal by SPR, i.e. affinity for F4 fimbriae, were planned to be tested for their inhibition effect on F4 positive ETEC attachment.

Two SPR experiments were attempted on the Biocore X system (GE Healthcare Life-Sciences, Uppsala, Sweden) using different sensor chips. The first experiment used a CM5 sensor chip (GE Healthcare Life-Sciences, Uppsala, Sweden) with 100nm thick carboxymethylated dextran covalently attached to a gold surface. Immobilization of F4ac fimbriae (100 µg/ml in 10mM acetate buffer pH 5.0) was performed using an amine coupling procedure according to the manufacturer's instructions. However, several attempts failed to immobilize sufficient amounts of purified F4ac on the surface of the sensor chip to reach the appropriate RU values (minimum 5000) for detection. Therefore in the second SPR experiment a streptavidin modified sensor chip, SAHC 1000M (Xantec Bioanalytics GmbH, Duesseldorf, Germany) and biotinylated F4ac fimbriae were used. The SAHC sensor chip is coated with bioinert polycarboxylate hydrogel matrix with streptavidin and allows immobilization of biotinylated ligands through a specific biotin-streptavidin interaction. Although this strategy seemed initially promising, SPR signal values obtained in the experimental setting never exceeded 2000 RU, which was not enough to detect significant differences in binding affinity between the positive and negative controls, therefore, the SPR experiments were terminated.

5 EVALUATION OF INHIBITION OF F4AC+*ESCHERICHIA COLI* ATTACHMENT WITH XANTHINE DEHYDROGENASE, BUTYROPHILIN, LACTADHERIN AND FATTY ACID BINDING PROTEIN

This chapter will be submitted for publication to the Veterinary Microbiology as the following manuscript:

Predrag Novakovic [*], Chandrashekhar Charavaryamath [*], Betty Lockerbie, Radhey S. Kaushik, Matthew E. Loewen, Beverly A. Kidney, Igor Moshynskyy, Chris Stuart, Elemir Simko

(* equal contribution)

5.1 Introduction

Post-weaning diarrhea (PWD) due to F4ac-positive enterotoxigenic *Escherichia coli* (ETEC) is an important cause of morbidity and mortality in weaned piglets [5]. Currently, PWD is controlled by various management strategies, use of antibiotics as feed supplements and/or immunization with vaccines containing fimbrial antigens. However, none of these control measures can completely eliminate PWD from modern swine production. In addition, continuous use of sub-therapeutic doses of antibiotics as feed supplements potentially leads to the emergence of genes encoding antimicrobial resistance in porcine microflora. These antimicrobial resistant genes may be incorporated by animal and human pathogens, potentially causing serious public health problems. Hence, there is a great demand to find alternative strategies for prevention and control of porcine post-weaning diarrhea (PWD). Many domestic animal species including pigs are born hypogammaglobulinemic and rely on sow's milk for the immune protection. Vaccination of sows efficiently protects piglets against ETEC infection only during nursing period. However, after weaning, ingestion of antibodies and other potentially protective

substances from sow's milk is terminated, and piglets become highly susceptible to ETEC infection. Since the immune system of neonatal piglets is relatively naïve, the current vaccination strategies at that age are not sufficiently effective for protection against PWD [21]. In addition to immunoglobulins, porcine milk also contains a variety of non-immunoglobulin substances that can also interfere with ETEC attachment to enterocytes.

Atroshi et al., reported that porcine MFGM can act as a target for binding of F4 positive *E. coli* [8]. Furthermore, it was demonstrated that porcine milk MFGM have the potential to inhibit binding of F4 fimbriae to porcine intestinal brush borders [9]. Recent studies revealed that certain proteins of porcine MFGM, such as lactadherin, butyrophilin, adipophilin, acyl-CoA synthetase and fatty acid binding protein have binding affinity for F4ac fimbriae of ETEC [11, 117, 153].

The purpose of this study was to isolate the previously identified F4ac-binding proteins from porcine MFGM and to determine if they can inhibit attachment of F4ac-fimbriae or F4ac positive *E. coli* to primary porcine enterocytes or IPEC-J2 cell line using competitive ELISA assay.

5.2 Material and Methods

5.2.1 IPEC-J2 cell line and culture conditions

The IPEC-J2 [undifferentiated porcine intestinal epithelial cell line derived from porcine jejunum, a kind gift from Dr. Pradip Maitii (Nutratechglobal, Winnipeg, MB, Canada)] cells were seeded on cell culture flask (T75cm^2, Corning, NY, USA) as described previously [154]. Briefly, IPEC-J2 cells were cultured and maintained in Dulbecco's Modified Eagle Medium

(DMEM)-Hank F12 (Gibco, Invitrogen Corporation, Grand Island, NY, USA) supplemented with 5% fetal calf serum (FCS, Atlanta Biologicals, Lawrenceville, GA, USA), penicillin (100 IU/ml), streptomycin (100 μg/ml) (Invitrogen Corporation, Grand Island, NY, USA), and 5 ng/ml of epidermal growth factor (Sigma Chemical Co., St. Louis, MD, USA). IPEC-J2 cells were maintained in a humidified incubator in an atmosphere of 5% CO_2 and 95% air at 37^o C. After 4 to 5 days of culturing, the confluent cell monolayer was briefly rinsed with 1x PBS pH 7.4 (Gibco, Invitrogen Corporation, Grand Island, NY, USA) to remove all traces of serum. Five hundred microliters of 0.25 % Trypsin-EDTA solution (Gibco, Invitrogen Corporation, Grand Island, NY, USA) was added to the flask and incubated at 37^{o}C. Cells were observed under an inverted microscope and incubation was continued with trypsin-EDTA until the cell monolayer was completely dispersed (usually within 7 to 10 min). The detached cells were collected and centrifuged at 200 × *g* for 5 min, resuspended in ice cold phosphate-buffered saline (PBS) (1.6 mM NaH_2PO_4, 9.4 mM Na_2HPO_4, 154 mM NaCl, pH 7.4) to a density of 2 x 10^6cells/ml before being used for the ELISA test. The continuous culture of the IPEC-J2 cells was maintained by seeding culture flasks at 1:4 or 1:5 ratios at each passage into T75cm^2 flasks.

5.2.2 Pig enterocyte isolation

The animal use protocol was reviewed and approved by the Animal Research Ethics Board (AREB) at the University of Saskatchewan and followed the principles established by the Canadian Council on Animal Care [155-156].

The isolation of fresh enterocytes was done by using a distended intestinal sac method as described previously [157]. Briefly, a six to eight day old piglet from Prairie Swine Center was euthanized with a halothane overdose (MTC Pharmaceuticals, Cambridge, ON, Canada) and

subsequent exsanguination. An 80-cm segment of jejunum was dissected, and rinsed with a pre-incubation buffer [PBS with 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mM dithiothreitol]. The caudal end was then clamped, and the segment was filled with isolation buffer (PBS with 1.5 mM EDTA, 0.2 mM PMSF, and 0.5 mM dithiothreitol) until fully distended. The cranial end was then clamped, and the distended jejunal segment was placed in pre-warmed saline in a 2 l beaker, kept on a shaking water bath at 37 ^{o} C. After 20 minutes of incubation [158], contents of the segment including isolated enterocytes were collected into a conical tube and centrifuged (400 x *g* for 3 min at 4^{o}C). The cell pellet obtained after centrifugation was resuspended in ice cold PBS and this washing step was repeated 4 times. Finally, the enterocytes were resuspended in ice cold PBS to a density of 2 x 10^{6} cells/ml before using in an ELISA test.

5.2.3 F4ac-positive *Escherichia coli*

F4ac-positive *Escherichia coli* (reference strain P97-2554B, O149:K91:F4ac) isolated from the intestine of a pig with diarrhea at the faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, QC was kindly provided by Dr. Musangu Ngeleka, Prairie Diagnostic Services, Inc., Saskatoon, SK.

5.2.4 Culture and use of F4ac-positive *Escherichia coli*

An overnight growth of bacterium in tryptic soy broth (TSB) was mixed in sterile glycerol to a final concentration of 15% glycerol and stored at – 20 ^{o} C for future use. A loop full of glycerol

stock culture was spread on a tryptic soy agar (TSA) plate with 5% sheep blood and incubated at 37 ^{o} C overnight. Next day, a single colony was inoculated into 30 ml of TSB and incubated at 37 ^{o} C with 150 rpm shaking over night. The following day, the bacterial suspension was centrifuged (3000 x *g*, for 30 minutes at 25 ^{o} C) and the pellet was resuspended in carbonate buffer (100 mM $NaCO_{3,}$ pH 8.2). Using a spectrophotometer (NanoDrop 2000C, Thermofisher Scientific, Wilmington, DE, USA), OD of the bacterial suspension was adjusted to 0.7 (A550 nm) against carbonate buffer as the blank value.

5.2.5 Isolation and identification F4ac fimbriae

F4ac fimbriae were isolated and purified according to a previously published method [143], the only modification being the introduction of filtration of solubilized F4ac fimbriae through 0.20µm pore size filters (Nylon Membrane 0.2µm 47 mm PK/100, Millipore Corporation, MA, USA) before each step of precipitation at pH 4 during the purification process to prevent aggregation [153]. The concentration of isolated fimbriae was determined with Quick Star Bradford protein assay (Bio-Rad Laboratories, Mississauga, Ont., Canada), the purity by Sodium Dodecyl Sulphate (SDS) Polyacrylamide Gel Electrophoresis (PAGE) separation and Coomassie staining and the identity by Western Blot technique using rabbit anti-F4ac polyclonal antibodies (Dr J.M. Fairbrother, Faculte de Medecine Veterinaire, Universite de Montreal, Saint-Hyacinthe, QB, Canada) as previously described [153]. The isolated F4ac fimbriae was stored at -20^{o}C in the PBS.

5.2.6 Isolation of xanthine dehydrogenase, butyrophilin, lactadherin and fatty acid binding protein

MFGM proteins were isolated from porcine milk fat according to previously published methods [144-145] with minor changes described previously [153].

Isolation of the four individual MFGM proteins of interest, namely, xanthine dehydrogenase (XDH), butyrophilin (BTN), lactadherin (LAD) and fatty acid binding protein (FABP) was done by electro-elution technique. Briefly, MFGM proteins were loaded in multiple lanes (100 µg/well) and separated in 12% polyacrylamide gel by SDS PAGE using a large electrophoretic apparatus (Protean II xi Cell, Bio-Rad Laboratories, Mississauga, Ontario, Canada) under constant current of 24 mA for 5 hours. After separation, the reference lane was cut off and the separated proteins were visualized by Coomassie staining as previously described [159].The Coomassie-stained reference lane was lined up with the rest of the unstained gel to cut the regions of unstained proteins corresponding to individual separated and Coomassie-stained proteins in the reference lane. Unstained gel regions containing individual MFGM proteins were sliced in 1-2 cm segments and loaded into an electro-elution system (Elutrap, Whatman, Dassel, Germany). After 8 hours of elution under 200 V in Tris-glycine buffer (25mM Tris, pH 8.3; 192mM glycine; 0.1% Sodium Dodecyl Sulphate), eluted individual proteins were collected. The remaining SDS was removed from isolated proteins by dialysis (72 hours, with changes of deionized water every 4 hours, at 4^{o} C) using cellulose dialysis tubing with 8 kDa cut-off (Spectra/Por, Spectrum Laboratories, Inc., CA, USA). Finally, purity of the individual proteins eluted from the 12% acrylamide gel was analyzed by Coomassie staining after SDS-PAGE separation, and the concentration was determined with Quick Star Bradford protein assay (Bio-Rad Laboratories, Mississauga, ON, Canada).

5.2.7 Microscopic examination of adherence of F4ac-positive *E. coli* to IPEC-J2 and pig enterocytes

IPEC-J2 cells or freshly isolated enterocytes (2 x 10^6 cells/ml, 100 µL/slide) were spread on microscopic glass slides, allowed to dry and stored at – 80 ^{o} C. Prior to use, the slides were allowed to thaw at room temperature and bacterial suspension (OD 0.7 at 550 nm) prepared in PBS containing 1% D-Mannose was added to the cells on the slide (200 µL/slide). Slides were kept gently shaking at room temperature for 1 hour, washed with PBS, dried, stained with Diff-quick and cover slip mounted. Entire area of slide was examined for bacterial attachment to the cells.

5.2.8 Coating of microtiter plates with IPEC-J2 and pig enterocytes

Immobilization of IPEC-J2 cells and fresh pig enterocytes was done as previously published [160]. Briefly, 100 µ1 of 1 M lysine solution (in distilled water) was added to each well of 96-well, flat-bottomed polystyrene microtiter plates (Gibco BRL, Canadian Life Technologies, Burlington, ON, Canada) and incubated for at least 10 minutes (up to 2 hours) at room temperature. After three washes with distilled water, 100 µ1 of a 1.25% glutaraldehyde solution (in distilled water) was added to each well. The plates were incubated for exactly 5 minutes and then they were washed 2 times with distilled water to activate the plates. PBS (100 µ1) was immediately added to each well, followed by 50 µl of cell suspension (2x 10^6 cells/ml, IPEC-J2 or freshly isolated pig enterocytes). The optimal numbers of cells for competitive ELISA were determined by checkerboard titration in direct ELISA, testing various numbers of cells with various numbers of biotinylated F4-positive *E. coli* (see Appendix, Figure 8-2). The cells were gently mixed and allowed to settle for 10 minutes at room temperature. The plates were then

centrifuged for 10 minutes at 650 x *g*. The supernatant (100 µl) was aspirated with caution, so as not to disrupt the cell monolayer. The plates were then dried overnight at 37°C and stored at room temperature until used (not longer than 10 days).

5.2.9 Biotinylation of F4ac-positive *E. coli* and F4ac fimbriae

F4ac-positive *Escherichia coli* was cultured and bacterial suspension with optical density of 0.7 at 550 nm in carbonate buffer as described above. 12.5 µ1 of reconstituted NHS-PEO_4 Biotin in distilled water (EZ-Link NHS-PEO-Biotinylation kit, Pierce Biotechnology, Rockfor, IL, USA) was added to 1 ml of the bacterial suspension, mixed and incubated for 2 hours at room temperature. The bacteria were then washed 3 times with PBS (3000 x *g*, 10 minutes at room temperature) and finally resuspended in PBS with 1% (wt/vol) D-mannose (Sigma Chemical Co., St. Louis, MD, USA) to the original volume (5 ml) just before use in the ELISA test. The optimal numbers of bacteria (2 x 10^8/ml of biotinylated F4ac-positive *E. coli*) for competitive ELISA were determined by checkerboard titration in direct ELISA, testing various numbers of biotinylated F4-positive *E. coli* with various numbers of porcine enterocytes (see Appendix, Figure 8-1).

Biotinylation of F4ac fimbriae was done according to the manufacturer instructions for biotinylation of proteins (EZ-Link NHS-PEO-Biotinylation kit, Pierce Biotechnology, Rockfor, IL, USA).

5.2.10 Competitive ELISA for quantification of inhibition of adherence of F4ac-positive *E. coli*

The competitive ELISA test for quantification of inhibition of F4ac-positive *E. coli* adherence to IPEC-J2 cells with isolated MFGM proteins was done as previously published [160] with few modifications. The unoccupied plastic spaces in a 96-well microtiter plate containing immobilized IPEC-J2 was blocked by adding 300 µl/well of a 5% solution of BSA in PBS. The plate was incubated for 3 hours at 37°C and washed 3 times in PBS (300 µl/well). Then, 1 ml of 2 x 10^8 biotinylated F4ac-positive *E. coli* in 1% PBS-D-mannose buffer had been pre-incubated with isolated xanthine dehydrogenase (5, 25 or 125 µg); butyrophilin (5, 25 or 125 µg); lactadherin (5, 25 or 125 µg), or fatty acid binding protein (5, 25 or 125 µg). Rabbit anti-F4ac polyclonal antibody was used as positive control (5, 25, or 125 µg of polyclonal serum proteins) (Dr J.M. Fairbrother, Faculte de Medecine Veterinaire, Universite de Montreal, Saint-Hyacinthe, QB, Canada), and rabbit anti-F6 polyclonal antibody (125 µg) (Dr J.M. Fairbrother, Faculte de Medecine Veterinaire, Universite de Montreal, Saint-Hyacinthe, QB, Canada) and lactalbumin (125 µg) (Gibco BRL, Canadian Life Technologies, Burlington, ON, Canada) as negative controls. In order to determine nonspecific binding, additional controls were also included, such as wells with only cells (immobilized IPEC-J2 or fresh enterocytes) without biotinylated F4ac-positive *E. coli,* and wells with PBS only without cells or biotinylated F4ac-positive *E. coli.* After 1 hour of incubation of biotinylated F4ac-positive *E. coli* with test reagents at room temperature with gentle shaking, 50 µl was added to each well of the microtiter plate for ELISA. The plate was incubated for 40 minutes at 37^{o}C, and subsequently washed 3 times with PBS. Then 100 µl of PBS was added into each well and the plate was heat-fixed for 10 minutes at 60-65°C. After that PBS was removed and horseradish peroxidase streptavidin conjugate (100

μl/well, 1:7000 dilution in PBS) (Vector Laboratories, Inc., Burlingame, CA, USA) was added to each well and incubated for 30 minutes at 37°C. The plate was washed 3 times with PBS, and 100 μl of TMB microwell peroxidase substrate system (KPL, Gaithersburg, MD, USA) was added to each well. The color was allowed to develop in the dark for 15 to 30 minutes and then stop solution (2N H_2SO_4, 100 μl/well) was added. ELISA optical density (OD) was measured at 450nm within 30 minutes with Spectra Max 340 PC Spectrophotometer operated by SoftMax Pro software (Molecular Devices Corporation, Sunnyvale, CA, USA). The OD of test wells was plotted after deducting the average OD of wells treated with PBS only in order to account for non-specific background color development.

The competitive ELISA test for quantification of inhibition of F4ac-positive *E. coli* adherence to pig enterocytes with isolated MFGM proteins was done as described for IPEC-J2 cells, with the exception that freshly isolated enterocytes were used to coat microtiter plates.

5.2.11 Competitive ELISA for quantification of inhibition of F4ac fimbriae attachment

The competitive ELISA for quantification of inhibition of F4ac fimbriae attachment to IPEC-J2 with MFGM proteins was performed similarly as described for F4ac-positive *E.coli*. This test was used to determine whether isolated MFGM protein mediated inhibition of F4ac-positive *E. coli* adherence to IPEC-J2 cells *in vitro* is because of the specific interaction between MFGM protein and F4ac fimbriae. The ability of biotinylated F4ac fimbriae to attach to IPEC-J2 cells and optimal concentration for competitive ELISA test were determined by direct ELISA, testing various concentrations of biotinylated F4ac fimbriae on IPEC-J2 coated microtiter plates (see Appendix, Figure 8-3). The concentration of 0.5 μg/ml of purified biotinylated F4ac fimbriae was determined as optimal and used in the competitive ELISA test as described for competitive

ELISA for quantification of inhibition of adherence of F4ac positive *E. coli* to IPEC-J2 cells with MFGM proteins. Briefly, 0.5 μg/ml (molar concentration, 18.52 nM) of biotinylated F4ac fimbriae was incubated with 5 μg/ml (66.67 nM), 25 μg/ml (333.33 nM), or 125 μg/ml (1.67 μM) of xanthine dehydrogenase; 5 μg/ml (151.51 nM), 25 μg/ml (757.57 nM), or 125 μg/ml (3.79 μM) of butyrophilin; 5 μg/ml (212.77 nM), 25 μg/ml (1.06 μM), or 125 μg/ml (5.32 μM) of lactadherin; and 5 μg/ml (769.23 nM), 25 μg/ml (3.85 μM), or 125 μg/ml (19.23 μM) of fatty acid binding protein for 1 hour at room temperature. The negative controls and other conditions of the test were the same as described above.

5.2.12 Statistical analysis

The data were analyzed for statistical significance ($P < 0.05$) by ANOVA with Tukey's multiple comparisons test (post-hoc test) using Prism 5 for Windows, version 5.04 (GraphPad Software, Inc., La Jolla, CA, USA).

5.3 Results

5.3.1 Adherence of F4ac-positive *E. coli* to IPEC-J2 cell and pig enterocytes

In order to assess the ability of each MFGM protein of interest to inhibit F4ac-positive *E. coli* adherence to porcine intestine, we employed an already established cellular system (IPEC-J2 cell line) [154] and confirmed that F4ac-positive *E. coli* adheres to both IPEC-J2 cells and pig enterocytes in a slide adherence assay (Figure 5-1).

5.3.2 Isolation of F4ac fimbriae from F4ac-positive *E. coli*

We isolated F4ac fimbriae from bacterial culture, analyzed its purity by SDS-PAGE separation and Coomassie staining and confirmed its identity by Western blot (Figure 5-2).

5.3.3 Isolation of MFGM proteins

We isolated xanthine dehydrogenase, butyrophilin, lactadherin, and fatty acid binding protein by electro-elution. To confirm purity of individual proteins we separated those by SDS-PAGE, and visualized by Coomassie blue stain (Figure 5-3).

5.3.4 Quantification of inhibitory effect of MFGM proteins on attachment of F4ac-positive *E. coli* to the IPEC-J2 cells

We performed three different experiments to analyze the inhibitory effect of MFGM proteins against F4ac-positive *E. coli* adherence by competitive ELISA. The first experiment consisted of Competitive ELISA test for quantification of inhibition of adherence of F4ac-positive *E. coli* to IPEC-J2 cells with various concentrations (5, 25 or 125 μg) of MFGM proteins in comparison to positive (rabbit anti-F4ac polyclonal antibody) and negative controls (rabbit anti-F6 polyclonal antibody and lactalbumin) and demonstrated that lactadherin, butyrophilin, and fatty acid binding protein inhibit adherence of F4ac-positive *E. coli* to the IPEC-J2 cells (Figure 5-4).

5.3.5 Quantification of inhibitory effect of MFGM proteins on attachment of F4ac-positive *E. coli* to the pig enterocytes

In the second experiment we tested biological relevance of our *in vitro* system IPEC-J2 by repeating Competitive ELISA test with freshly isolated pig enterocytes and obtained very similar results (Figure 5-5).

5.3.6 Quantification of inhibitory effect of MFGM proteins on attachment of F4ac fimbriae to the IPEC-J2 cells

Finally, in order to confirm that inhibition of F4ac-positive *E. coli* adherence to IPEC-J2 cell line *in vitro* is because of the specific interaction between MFGM protein and F4ac fimbriae, we designed the Competitive ELISA test for quantification of inhibition of F4ac fimbriae attachment to IPEC-J2 cells and confirmed that inhibition of attachment is mediated by interaction between individual MFGM proteins and F4ac fimbriae (Figure 5-6). Based on comparative inhibitory molar concentrations of MFGM proteins and F4ac, it appeared that 5 molecules of LAD, 50 molecules of BTN, and 200 molecules of FABP were needed to inhibit attachment of one molecule of F4ac fimbriae to IPEC-J2 cells.

5.4 Discussion

In the current study, we demonstrated that butyrophilin, lactadherin and fatty acid binding protein interfere with attachment of F4ac-positive *E. coli* to the IPEC-J2 cells or porcine enterocytes. The inhibitory effect exhibited by these MFGM proteins appears to be in a dose-dependent manner and due to a specific interaction with F4ac fimbriae.

It has long been known that porcine milk fat globule surrounded by milk fat globule membrane (MFGM) binds to F4-positive *E. coli* [8]. We demonstrated previously by two independent techniques (overlay Western blot and affinity chromatography) that acyl-CoA synthetase 3, butyrophilin, adipophilin, lactadherin, and fatty acid binding protein 3 have binding affinity for F4ac fimbriae *in vitro* [153]. In this study we confirmed not only that they interact with F4ac fimbriae, but they also exhibit inhibitory effects against binding of F4ac-positive *E. coli* to enterocytes and non-transformed porcine jejunal epithelial cell line (IPEC-J2) *in vitro*.

Lactadherin is a 46 kDa peripheral glycoprotein, and a major protein on MFGM. Lactadherin has an N-terminal epidermal growth factor (EGF)-like domain, which mediates binding to $\alpha_v\beta_5$ integrin [128]. Multiple functions of lactadherin have been reported so far. Lactadherin is involved in cellular adhesion, neovascularization and clearance of apoptotic cells [119, 129, 161]. Human lactadherin is associated with protection of human infants against rotaviral infection [115]. Porcine lactadherin was reported to have high affinity for F4ac fimbriae [117] [11] and able to prevent binding of F4ac positive *E. coli* to intestinal villi *ex vivo* [11]. Consistent with the previous report [11], lactadherin was found in this study to inhibit the attachment of F4ac positive *E. coli* to primary enterocytes and a jejunal cell line and attachment of purified F4ac fimbriae to a jejunal cell line. Compared to butyrophilin and fatty acid binding protein, lactadherin appears to be the most potent inhibitor of *E. coli* attachment to enterocytes. Based on comparative inhibitory molar concentrations of lactadherin and F4ac, it seems that a minimum of 5 molecules of lactadherin is needed to inhibit binding of 1 molecule of F4ac fimbria to a jejunal cell line.

Butyrophilin is the most abundant protein of MFGM [162]. It is a type I membrane glycoprotein and belongs to the immunoglobulin superfamily [118]. Milk fat secretion is mediated by a complex of butyrophilin, xanthine dehydrogenase and adipophilin [119]. The biological importance of butyrophilin has not been defined so far. We previously reported that it has a binding affinity to F4ac fimbriae *in vitro*, and here we confirmed that it inhibits F4ac positive *E. coli* attachment to enterocytes by interaction with F4ac fimbriae.

Fatty acid binding protein of MFGM is a 13 kDa protein [10]. Initially it was known as mammary-derived growth inhibitor, because it inhibited growth of mammary carcinoma cells [136]. Fatty acid binding protein in MFGM consists of a mixture of heart type and adipocyte type fatty acid binding protein [163]. Bovine mammary fatty acid binding protein does not appear to be glycosylated. The function of milk fatty acid binding protein is not yet clear [10]. Previous studies demonstrated that fatty acid binding protein from porcine milk interacts with F4ac fimbriae of ETEC *in vitro* [11, 117]. In this study we demonstrated that fatty acid binding protein inhibits F4ac positive *E. coli* attachment to enterocytes and IPEC-J2 cell line.

Xanthine dehydrogenase is present in many mammalian tissues, but it is a major constituent of the MFGM. Xanthine dehydrogenase is a complex enzyme comprising of two identical 147 kDa subunits [131], which catalyzes the oxidation of purines to uric acid by addition of oxygen from H_2O [10]. Xanthine dehydrogenase has been suspected to play an important role during the secretion of milk fat, facilitated by formation of a complex with butyrophilin and adipophilin [164]. Reactive oxygen species generated by xanthine dehydrogenase may also act as antibacterial components. In our previous study, xanthine dehydrogenase was isolated by F4ac-affinity chromatography together with other F4ac-fimbrial binding proteins, but its interaction with F4ac fimbriae was not detected with overlay Western blot [117, 153]; accordingly, we

suspected that the identification by affinity chromatography of xanthine dehydrogenase as a F4ac-binding protein was a false positive result. This was further confirmed in the current study by evidence that xanthine dehydrogenase did not inhibit attachment of F4ac positive *E. coli* to enterocytes or IPEC-J2 cell line nor purified F4ac fimbriae to IPEC-J2 cell line.

The inhibition of attachment of 0.5 µg/ml of F4ac fimbriae to IPEC-J2 cells with various concentrations of candidate MFGM proteins was tested in Competitive ELISA. Results suggested that only 5, 25 or 125 µg/ml of lactadherin and 25 or 125 µg/ml of butyrophilin and fatty acid binding protein were significant competitors. When molar concentrations of MFGM proteins were compared with F4ac fimbriae molarity, it appeared that 5 molecules of lactadherin, 50 molecules of butyrophilin, and 200 molecules of fatty acid binding protein were needed to inhibit attachment of one molecule of F4ac fimbria to IPEC-J2 cells.

In conclusion, lactadherin, butyrophilin and fatty acid binding protein reduce attachment of F4ac-positive *E. coli* to primary enterocytes and jejunal cell line (IPEC-J2) *in vitro*. The mechanism of this inhibition was not determined, but previous studies on lactadherin demonstrated that the glycosylated portion of lactadherin played an important inhibitory role and served as receptor analogues for F4ac fimbriae [11]. Future studies will be needed to determine the inhibitory importance of glycan portions of butyrophilin and fatty acid binding protein, and the potential importance of MFGM proteins in prevention of enteric colibacillosis *in vivo*.

Acknowledgements

This work was supported by Saskatchewan Agriculture Development Fund (20100103) and WCVM Canadian Vitamins Class Action Settlement. The authors would especially like to thank to Dr. Wojciech Dawicki for his useful advice in developing the ELISA test.

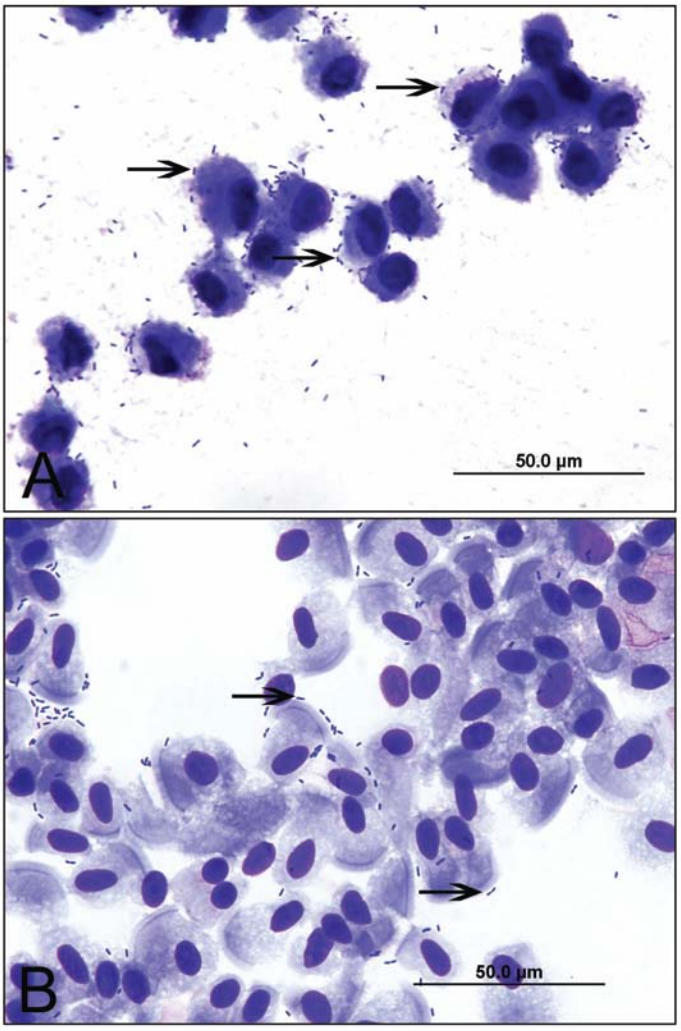

Figure 5-1 Adherence of F4ac-positive *E. coli* to IPEC-J2 cells and pig enterocytes. Microscopic examination shows *in vitro* adhesion of F4ac-positive *E. coli* to IPEC-J2 cells (A) and freshly isolated pig enterocytes (B) stained with Diff-quick stain (100x oil immersion objective).

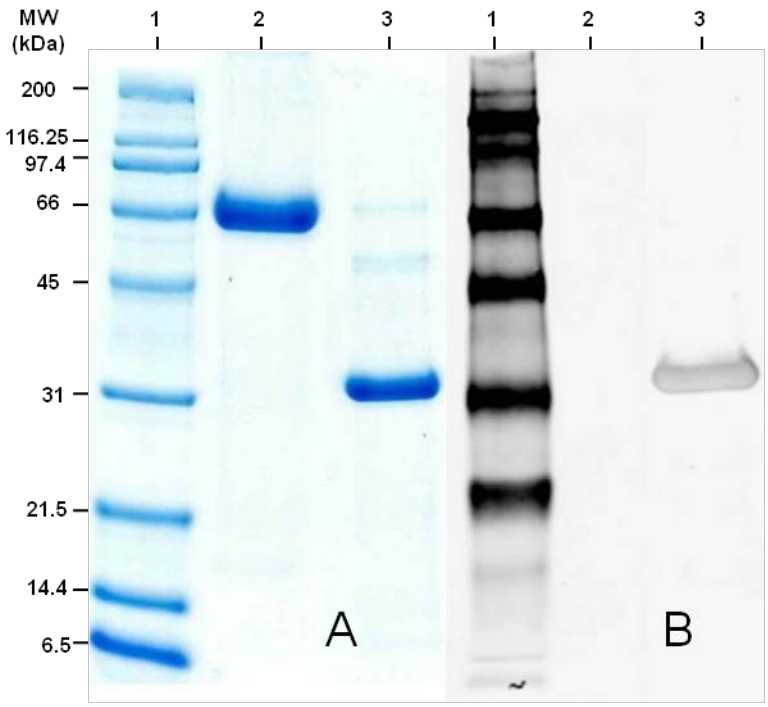

Figure 5-2 Isolation of F4ac fimbriae from F4ac-positive *E. coli*. F4ac fimbriae isolated from F4ac-positive *E. coli*, subjected to (A) 12% SDS-PAGE, and visualized by Coomassie Blue staining (B) and detected by Western blotting using primary rabbit anti-F4-polyclonal antibodies and secondary anti-rabbit Cy5 antibodies (Lane 1 – (A) Molecular weight markers; (B) ECL Flex Rainbow Molecular Weight Markers; Lane 2 - Bovine serum albumin as negative control; Lane 3- isolated F4ac fimbriae).

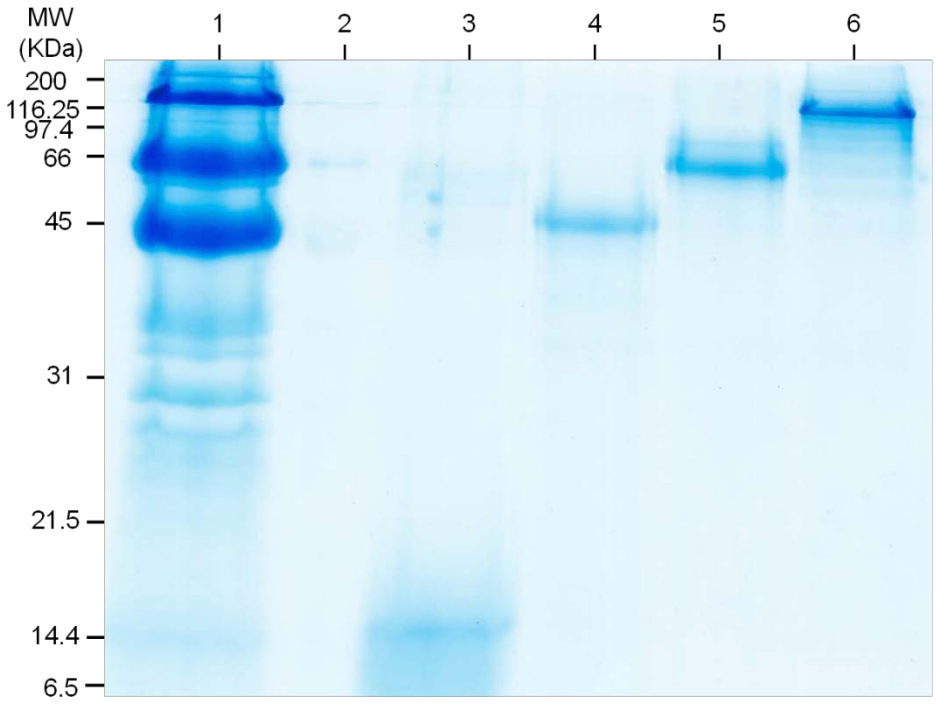

Figure 5-3 Isolation of MFGM proteins. Milk Fat Globule Membrane (MFGM) proteins isolated from porcine milk, subjected to 12% SDS-PAGE separation, and visualized by Coomassie Blue staining (Lane 1 – MGFM proteins; Lane 3 – Electroeluted fatty acid binding protein; Lane 4 – Electroeluted lactadherin; Lane 5 – Electroeluted butyrophilin; Lane 6 – Electroeluted xanthine dehydrogenase)

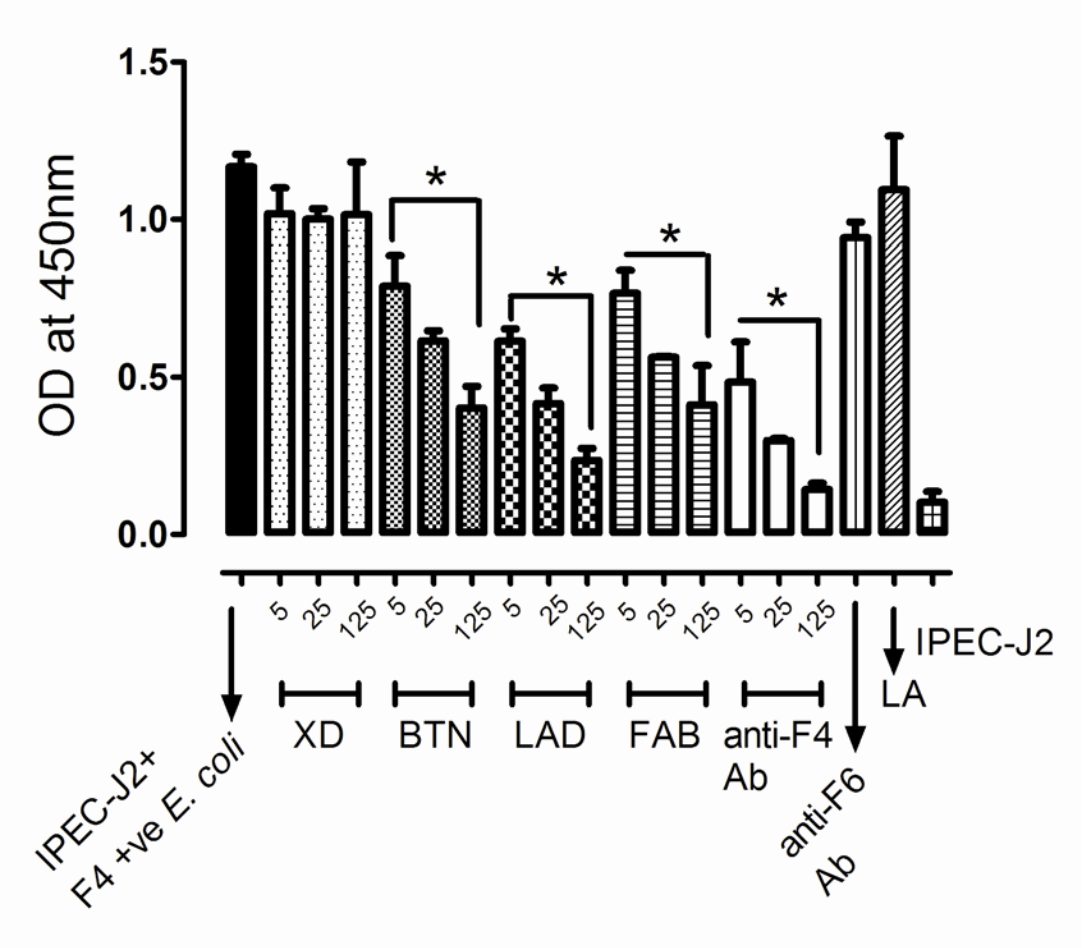

Figure 5-4 Quantification of inhibitory effect of MFGM proteins against attachment of F4ac-positive *E. coli* to the IPEC-J2 cells. Inhibition of F4ac-positive *E. coli* adherence to IPEC-J2 cells with various concentrations (5, 25 or 125 μg) of xanthine dehydrogenase (XD), butyrophilin (BTN), lactadherin (LAD), fatty acid binding protein (FAB); positive (rabbit anti-F4ac polyclonal antibody) and negative (rabbit anti-F6 polyclonal antibody and lactalbumin [LA]) controls. Only treatments marked with asterisk (*) are significantly different ($P < 0.05$) from both untreated (IPEC-J2 + $F4^+$ *E. coli*) and negative controls (LA and anti-F6 Ab).

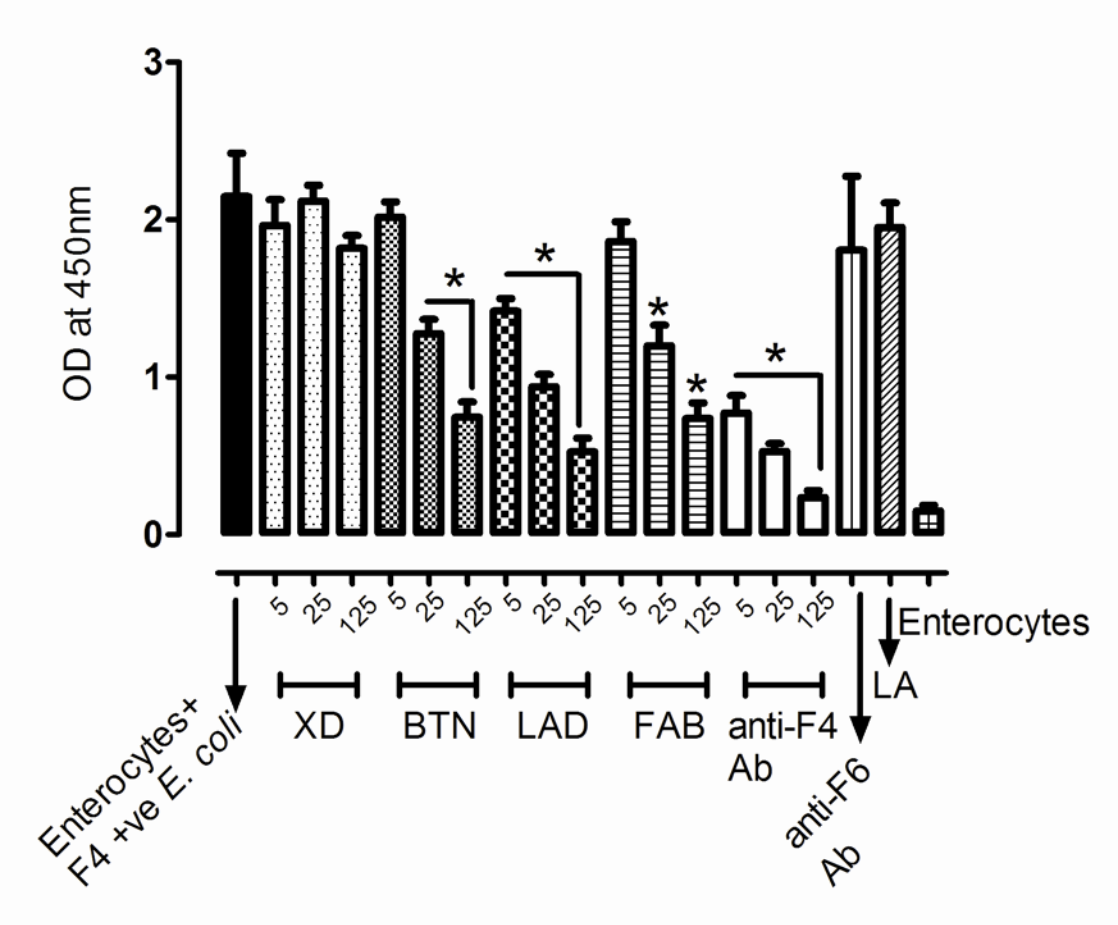

Figure 5-5 Quantification of inhibitory effect of MFGM proteins against attachment of F4ac-positive *E. coli* to the pig enterocytes. Inhibition of F4ac+*E.coli* adherence to freshly isolated pig enterocytes with various concentrations (5, 25 or 125 μg) of XD, BTN, LAD, FAB; positive (rabbit anti-F4ac polyclonal antibody) and negative (rabbit anti-F6 polyclonal antibody and LA) controls. Only treatments marked with star (*) are significantly different ($P < 0.05$) from both untreated (indicate as in the previous figure) and negative controls (indicate as in the previous figure).

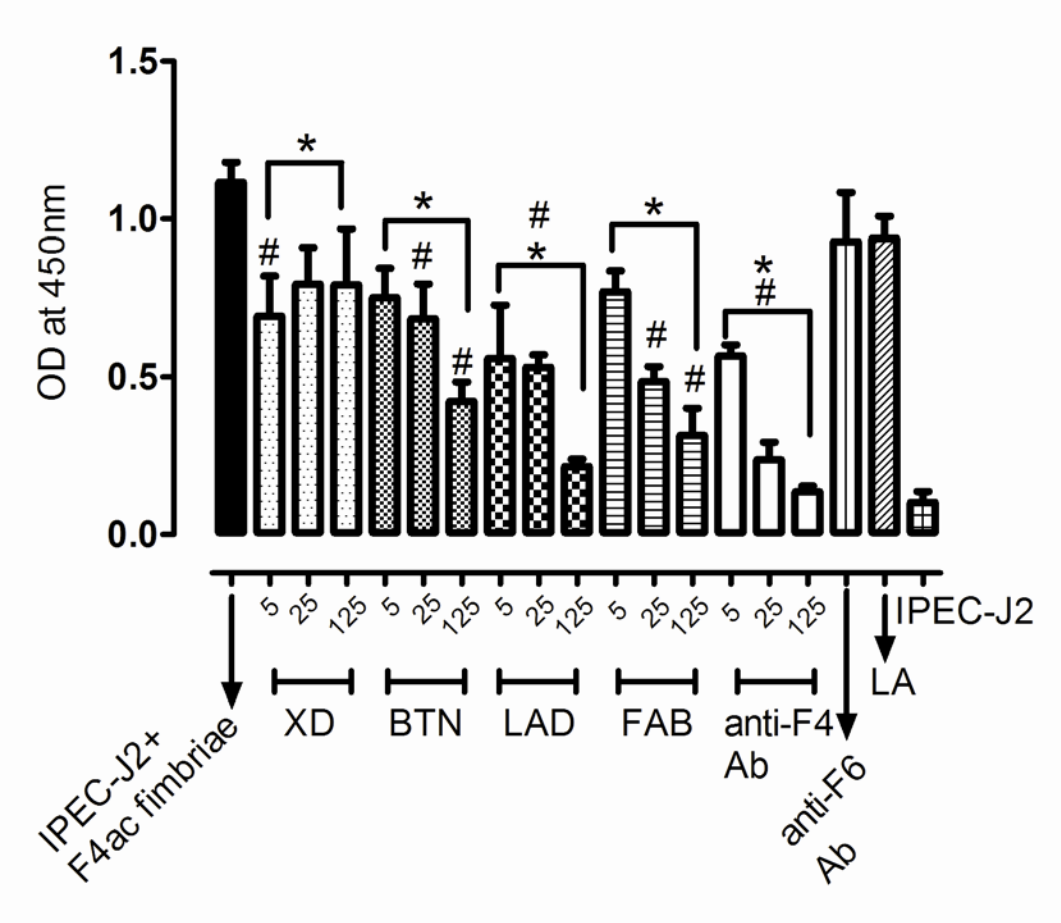

Figure 5-6 Quantification of inhibitory effect of MFGM proteins against attachment of F4ac fimbriae to the IPEC-J2 cells. Inhibition of F4ac fimbriae attachment to IPEC-J2 cells with various concentrations (5, 25 or 125 μg/ml) of XD, BTN, LAD, FAB; positive (rabbit anti-F4ac polyclonal antibody) and negative (rabbit anti-F6 polyclonal antibody and LA) controls. Treatments marked with asterisk (*) are significantly different ($P < 0.05$) from untreated (IPEC-J2 + F4ac fimbriae) control and treatments marked with (#) are significantly different ($P<0.05$) from negative controls (LA and anti-F6 Ab).

6 GENERAL DISCUSSION

The aims of the studies presented in this thesis were to isolate and characterize F4ac binding proteins from porcine MFGM, and evaluate their potential inhibitory effect on F4ac positive *E. coli* attachment *in vitro*. The experiment with affinity chromatography purification of F4ac binding proteins from MFGM presented in Chapter 4, revealed that xanthine dehydrogenase, acyl-CoA synthetase 3, butyrophilin, adipophilin, lactadherin, and fatty acid binding protein are binding to F4ac fimbriae coupled to activated Sepharose. Results generated by a competitive ELISA experiment presented in Chapter 5, demonstrated that butyrophilin, lactadherin and fatty acid binding protein inhibited adherence of F4ac *E. coli* to intestinal epithelial cells *in vitro*, but xanthine dehydrogenase did not. These results are consistent with the previous two studies conducted by our research group [11, 117].

The affinity chromatography experiment for isolation and purification of F4ac binding proteins from MFGM was adapted from previously published studies [11, 143]. The intention was to create *in vitro* experimental conditions biologically relevant to those required for pathogenesis of F4ac positive ETEC associated with post-weaning diarrhea. More specifically, our aim was to simulate the condition, where F4ac fimbriae mediated attachment enables *E. coli* bacteria to withstand the forces of intestinal peristalsis, by using a fast protein liquid chromatography (FPLC) system with controlled peristaltic flow. Of course, this approach did not take into consideration many other important factors present in the porcine intestine, which consequently might impact the biological relevance and interpretation of generated data. Nevertheless, affinity chromatography intended to offer independent verification of the results obtained in a previous study [117] that tested the interaction between MFGM proteins and F4ac fimbriae under complex chemical conditions associated with two-dimensional SDS PAGE.

Availability of simple and relatively rapid procedures for purification of sufficient amounts of F4ac fimbriae (50mg per 10ml column) [143] and MFGM proteins [144-145] made these experiments feasible. The inclusion of negative (control) CnBr-activated Sepharose 4B column together with an experimental column containing F4ac fimbriae covalently coupled to CnBr-activated Sepharose 4B had the purpose of comparing and subsequently excluding MFGM proteins that bind to Sepharose media, but not to F4ac fimbriae. Extensive wash (10 column volumes) was used to remove MFGM proteins that bound nonspecifically to the affinity matrix and/or ligand. Low (1M NaCl) and high (pH=2.5) affinity elutions were used to isolate proteins bound to F4ac fimbriae coupled to activated Sepharose.

The results demonstrated significant differences in amounts of MFGM proteins eluted from the negative control column and the experimental column with F4ac fimbriae. Fractions of proteins eluted from the negative control by 1M NaCl and low pH elutions were almost undetectable (<0. 1µg/ml per affinity chromatography run) as determined with Quick Star Bradford protein assay (Bio-Rad Laboratories, Mississauga, ON). Due to very low amounts of proteins they were not submitted for nanoLC-MS/MS identification. On the other hand, considerable amounts of proteins were eluted from the experimental column with F4ac fimbriae by both types of elutions (1mg/ml per affinity chromatography run). F4ac-fimbrial binding MFGM proteins eluted by 1M NaCl and low pH conditions were separated by 1D SDS PAGE, stained with Commassie (Chapter 4, Figure 4-5), excised, and identified by nanoLC-MS/MS as xanthine dehydrogenase, acyl-CoA synthetase 3, butyrophilin, adipophilin, lactadherin, fatty acid binding protein and lactotransferrin.

Affinity chromatography results were consistent with those obtained previously by Overlay Western blot technique [117]. Even though the experimental conditions used in affinity chromatography and overlay Western blot experiments differed markedly, both studies identified the following proteins that had binding affinity for F4ac fimbriae: acyl-CoA synthetase 3, butyrophilin, adipophilin, lactadherin, and fatty acid binding protein. In the overlay Western blot technique MFGM proteins were exposed to chemical treatment that included reducing agent, DTT and denaturing detergent, SDS. These reagents have ability to destroy quaternary, tertiary and secondary protein structure and reduce it to its primary structure [146]. On the other hand, experimental conditions of affinity chromatography did not change quaternary, tertiary or secondary structures of proteins from MFGM; hence, the proteins were in their native form from the moment of their reaction with F4ac fimbriae.

There are two possible explanations for these findings. The first explanation is that primary structures of MFGM proteins are playing an important role in interaction with F4ac fimbriae. Another explanation suggests that this mechanism is strictly based on lectin-carbohydrate interaction. Evidence that strongly supports this possibility is the example of human lactadherin. This glycoprotein inhibits binding of human rotavirus to their target cells and consequently prevents infant diarrhea. When the terminal sialic acid from the carbohydrate moiety of this glycoprotein was removed, the inhibitory activity for rotavirus was reduced. This strongly suggested that glycan residues were responsible for binding to rotavirus [115]. Additionally, when porcine lactadherin was treated with metaperiodate, it lost its ability to reduce binding of F4ac positive *E. coli* to intestinal villi *ex vivo* [11]. Periodate oxidation was used to confirm that the interaction between lactadherin and F4ac fimbriae is carbohydrate-lectin based. However, this procedure can potentially be damaging not just to carbohydrates, but also to proteins, and it

may introduce potential false results. Partial or complete inactivation of the antibodies may occur under the periodate oxidation, particularly if the easily oxidizable amino acids are essential for antigen binding [147]. Nevertheless, if glycan moieties are confirmed as binding sites on MFGM proteins for F4ac fimbriae, their characterization will become vital for development of a new strategy for prevention of PWD.

At the present time, only bovine lactadherin is known to contain both N- and O- linked glycans. N-linked glycans are comprised of two oligosaccharide structures, e.g. typical high-mannose containing (Manα1 → 6 (Manα1 → 3) Manβ1 → 4GlcNAcβ1 → 4GlcNAc) and GalNAcβ1 → 4GlcNAc sequence [10]. Interestingly, monosaccharide blocking studies indicated that terminal *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc) and galactose (Gal) may play a central role in the interaction of the F4 fimbriae with intestinal brush border receptors [148]. In contrast, glycosylation of porcine LAD and other porcine MFGM proteins remain completely unknown.

Xanthine dehydrogenase and lactotransferrin were a new finding in the affinity chromatography study. Given that the affinity chromatography experiment was based on use of native MFGM as the analyte, it was not clear whether this interaction was genuine or not. Therefore, we planned to purify this protein from MFGM and test its capacity to bind F4ac *in vitro*. The isolation of lactotransferrin, much better known as lactoferrin, from low pH eluate was completely unanticipated, because this soluble protein is frequently associated with secretory fluids, particularly skim milk. We believed that there are two possible explanations for this finding. The first explanation was that this lactoferrin is a regular part of MFGM, or so called membrane bound lactoferrin, a different entity from the one present in fluids, described so far only for human MFGM [150-151]. The second explanation is possible contamination of isolated

MFGM proteins with skim milk proteins, i.e. lactoferrin, which may have occurred during the washing of milk fat in the isolation procedure. However, the absence of other more abundant F4ac-binding proteins of skim milk (i.e. β casein) in both affinity chromatography eluates indicates that contamination is unlikely.

The second goal of this study was very challenging at the beginning because of several difficulties. Isolation of each individual protein from MFGM was a very complex task, due to lack of available techniques for purification. A few purification strategies were explored (e.g. phase separation with Triton X-114, etc.), but finally the decision was made to use the electro-elution technique to isolate individual MFGM proteins that were previously separated by SDS PAGE. Another challenge of this study was the establishment of the competitive inhibition assay to test individual MFGM proteins for their ability to inhibit attachment of F4ac positive *E. coli*. The *in vitro* villous adhesion assay has been the most commonly used and described assay for this type of studies in the literature. However, after taking into account all advantages and disadvantages associated with this assay, it was decided to employ enzyme-linked immunosorbent assay (ELISA) for this study. In general, ELISA systems are highly versatile, rapid, sensitive, and quantitative techniques for estimation of attachment of microorganism *in vitro* [165]. A previously described ELISA system [160] has been modified for the quantification of attachment of F4ac positive *E. coli* and F4ac fimbriae. The innovation, compared to the old system, which used porcine enterocytes, was an introduction of a new cellular system, IPEC-J2 cell line. IPEC-J2 is a non-transformed columnar epithelial cell line isolated from neonatal piglet mid-jejunum by Helen Berschneider at the University of North Carolina in 1989 [166]. Initially, this cell line was used as a model of the porcine small intestine in a study of the pathogenesis of porcine proliferative enteropathy [167]. After that, interest for IPEC-J2 has begun to grow

particularly in studies that investigate epithelial innate immune responses to microorganisms [168]. *Salmonella enterica* and *Escherichia coli* were most commonly used with IPEC-J2. IPEC-J2 cells can be invaded by *S. enterica* serovars, *S. typhimurium* and *S. choleraesuis* [169]. A particularly important fact for this study was that the IPEC-J2 cell line was widely used in adherence studies of pathogenic strains of *E. coli* [168]. It was confirmed that enteropathogenic *E. coli* induces attaching and effacing lesions on IPEC-J2 cells [169]. Several studies have demonstrated that F4 positive ETEC adhere to IPEC-J2 cells [154, 170]. Even more, it was confirmed that purified F4 fimbriae were capable of binding to IPEC-J2 cells [154, 170]. On the other hand, there are indications that IPEC-J2 cells do not express the F18 specific receptor, and F18 positive ETEC strains do not bind to these cells [154, 171]. Porcine ETEC strains were shown to bind to porcine gut epithelial IPEC-J2 better than to human-derived intestinal INT-407 cells, which confirms also the host specificity of this cell line [154]. Based on all these features of the IPEC-J2 cell line, it was decided to use them for the competitive inhibition study. It is believed that they significantly improved reproducibility of results and prevented possible variations due to the phenotypic or individual differences between pigs in this study. Results of competitive ELISA presented in Chapter 5 of this thesis confirmed that lactadherin isolated from porcine MFGM inhibits adherence of F4ac positive *E. coli* to porcine enterocytes *in vitro* more efficiently than any other porcine MFGM protein tested. This finding strongly supports and validates results obtained in previous studies [11, 117]. This study also demonstrated the inhibition effects of butyrophilin and fatty-acid binding protein. Xanthine dehydrogenase did not demonstrate a significant inhibitory effect on adherence of F4ac positive *E. coli in vitro*, even though it was isolated as an F4 binding protein in the affinity chromatography experiment. This might be explained by the fact that, in the native MFGM, this protein is complexed with

butyrophilin, which was undoubtedly confirmed to interact with F4ac fimbriae. The competitive ELISA technique was found to be a fast, reliable and sensitive method for testing inhibitory effects of proteins against attachment of F4ac positive *E. coli*, and F4ac fimbriae to enterocytes.

6.1 Conclusions/Future directions

In conclusion, the studies presented in this thesis demonstrated that porcine MFGM is composed of several proteins that have binding affinity for F4ac fimbriae and that some of them have the ability to inhibit attachment of F4ac positive *E. coli* to porcine enterocytes *in vitro*. They are most likely not key factors of innate protection against ETEC in porcine milk, but in conjunction with other substances may contribute to the defense against infection.

Future research work in this area has the potential to evolve into several different directions. One may be focused on investigation of mechanisms that are responsible for the inhibitory effect of MFGM proteins on F4ac positive *E. coli* attachment. For example, identification and characterization of glycan portions of porcine LAD and other F4-binding MFGM proteins may prove to be a very useful approach. Several experimental approaches could be employed for that purpose. Lectin binding studies could be used to identify either an oligosaccharide sequence of interest or the carbohydrate composition of these glycoproteins. Moreover, sequential enzyme digestion studies could also identify potential binding sites on the glycan moieties of LAD and other MFGM proteins of interest. For example, different glycoside hydrolases have the ability to remove specific terminal monosaccharide or oligosaccharide residues from glycoproteins, which could be later evaluated for their inhibitory activity against the bacterial attachment in a competitive inhibition assay. Subsequently, based on loss of the inhibitory effects associated

with carbohydrate hydrolysis, the structure of the functional carbohydrate moiety of the particular glycoprotein could be determined.

Another direction for future research may be feasibility studies for the application of MFGM proteins in prevention of PWD in pigs. However, this would require clinical trials with an investigation of effects of these proteins on ETEC infection *in vivo*. Even if there were beneficial effects, the commercial feasibility of production of porcine MFGM proteins is highly unlikely. Alternative sources of MFGM proteins, such as those from bovine milk would have to be considered. Bovine MFGM proteins are found in significant quantities in buttermilk, a commercially available byproduct of industrial dairy production of butter [172]. However, both *in vitro* and *in vivo* studies are needed first to determine if bovine MFGM proteins have an inhibitory effect on F4 positive ETEC attachment.

Considering all possible options, the best approach would be to discover specific carbohydrate receptor analogs that could be commercially produced and added as non-antibiotic feed additives for the prevention of porcine PWD.

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8 APPENDIX

The Competitive ELISA system, previously described by Ofek et al., 1986, was optimized, before it was used in this study (Chapter 5). In other words, the working concentration of each component of the test was assessed. More specifically, this assessment was done through the use of checkerboard titrations (CBTs). The process of CBTs involves the dilution of two reagents (i.e. porcine enterocytes and biotinylated F4ac-positive *E. coli*) against each other to examine the activities inherent at all the resulting combinations. In the first step, porcine enterocytes were attached to 96-well polystyrene microtiter plates as described in Chapter 5 in a series of two fold dilutions starting from row A to G. Row H received diluent only (PBS). The second step of titration involved making a series of two fold dilutions of biotinylated F4ac-positive *E. coli* from column 1 to 11. Column 12 received only PBS. After incubation and washing, the color was developed as described in Chapter 5. The OD was measured at 450 nm and results were plotted as follows.

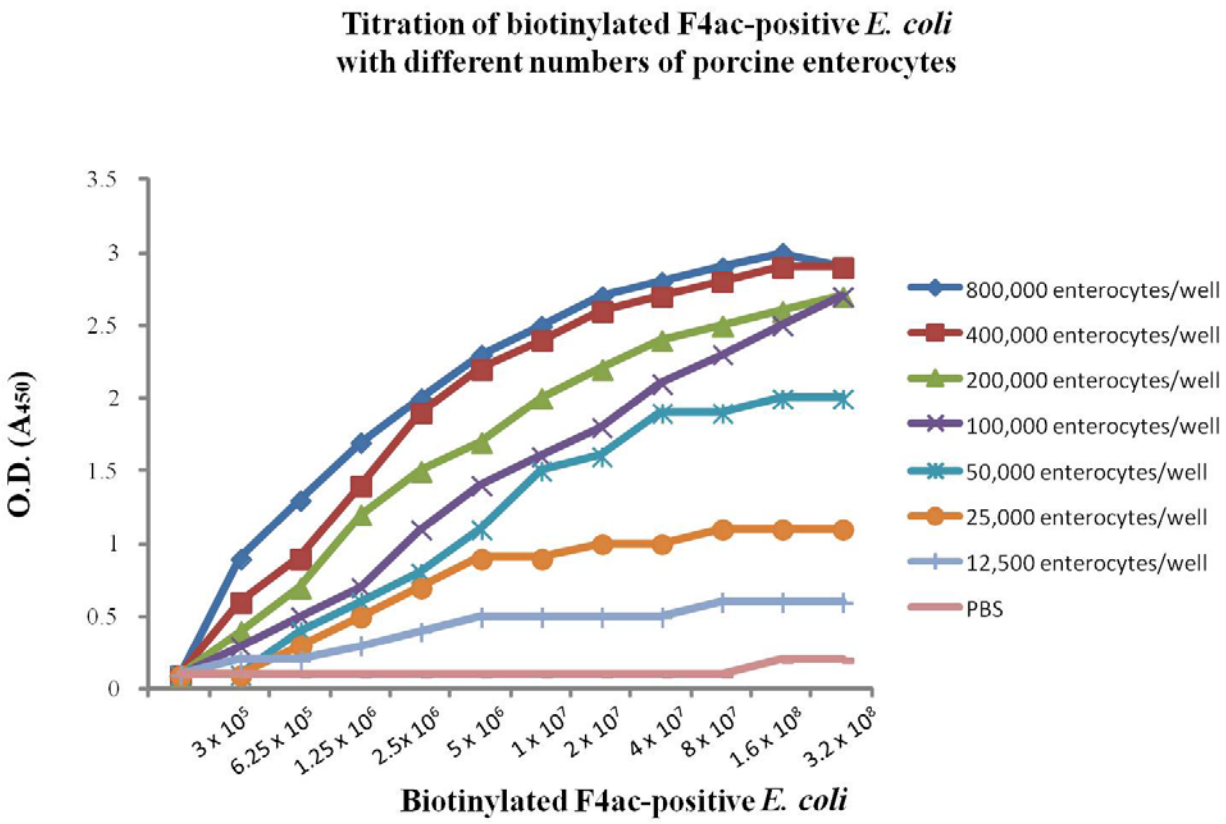

Figure 8-1 OD values obtained relating different numbers of biotinylated F4ac-positive *E. coli* per well to different numbers of porcine enterocytes per well of microtiter plate. Curves show titration of biotinylated F4ac-positive *E. coli* at different dilutions of porcine enterocytes.

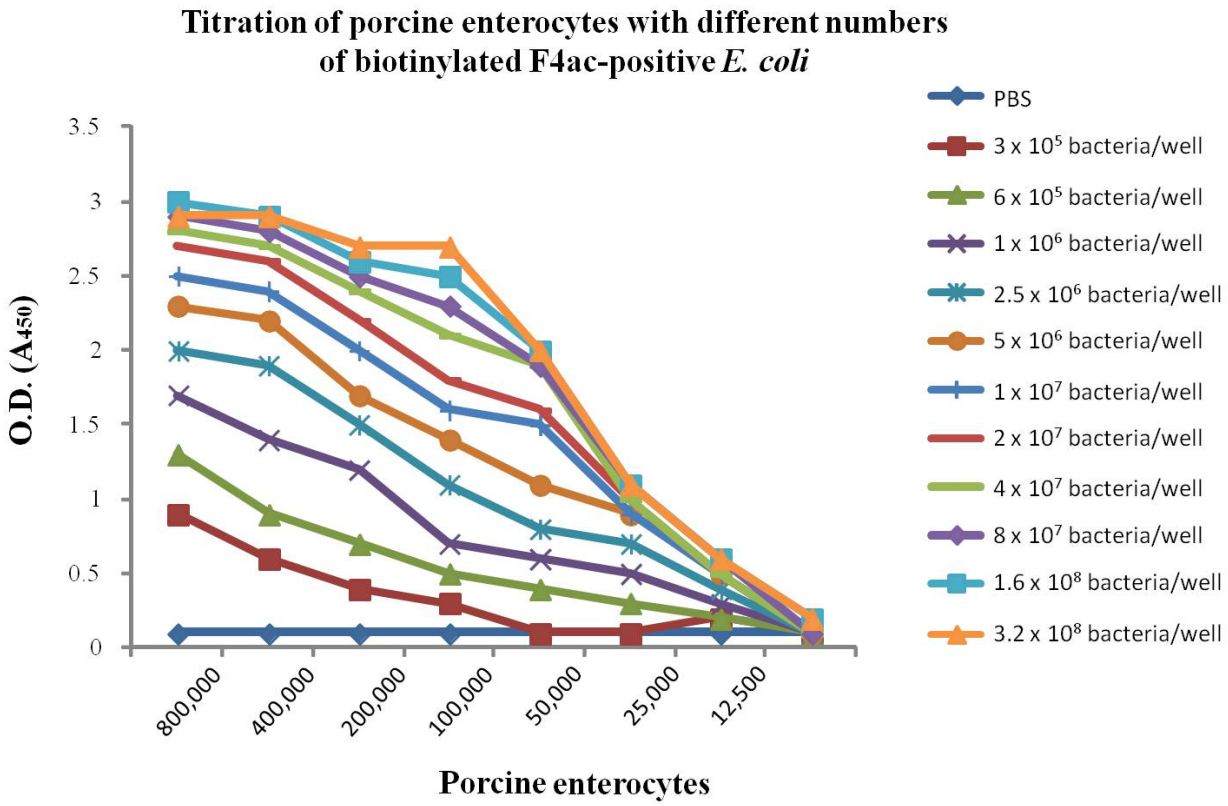

Figure 8-2 OD values obtained relating different numbers of porcine enterocytes per well to different numbers of biotinylated F4ac-positive *E. coli* per well of microtiter plates. Curves show titration of porcine enterocytes at different dilutions of biotinylated F4ac-positive *E.coli* per well of microtiter plate.

The results of CBTs test revealed that 1×10^7 of biotinylated F4ac-positive *E. coli* per well and 100,000 porcine enterocytes showed the best correlation of obtained OD values (curve that graphically presents almost linear fitting). Additionally, obtained OD values for this number of bacteria and enterocytes range between 1.5-1.7 OD units, which met the requirements for obtaining accurate result in ELISA test (Crowther, 2009). Finally these results were entirely in agreement with the conditions of ELISA test protocol previously described by Ofek et al., 1986.

When optimal number of 100,000 enterocytes per well for ELISA test was determined, this number of cells was used for assessment of optimal concentration of biotinylated F4ac fimbriae for Competitive ELISA test for quantification of inhibition of attachment of F4ac fimbriae to IPEC-J2 cell (Chapter 5). 100,000 IPEC-J2 cells were coated on the 96-well polystyrene microtiter plate and incubated with biotinylated F4ac fimbriae in a series of two fold dilutions starting from column 1 to 12. The same procedure for development of color and reading was repeated as in Chapter 5 and OD values were plotted as follows:

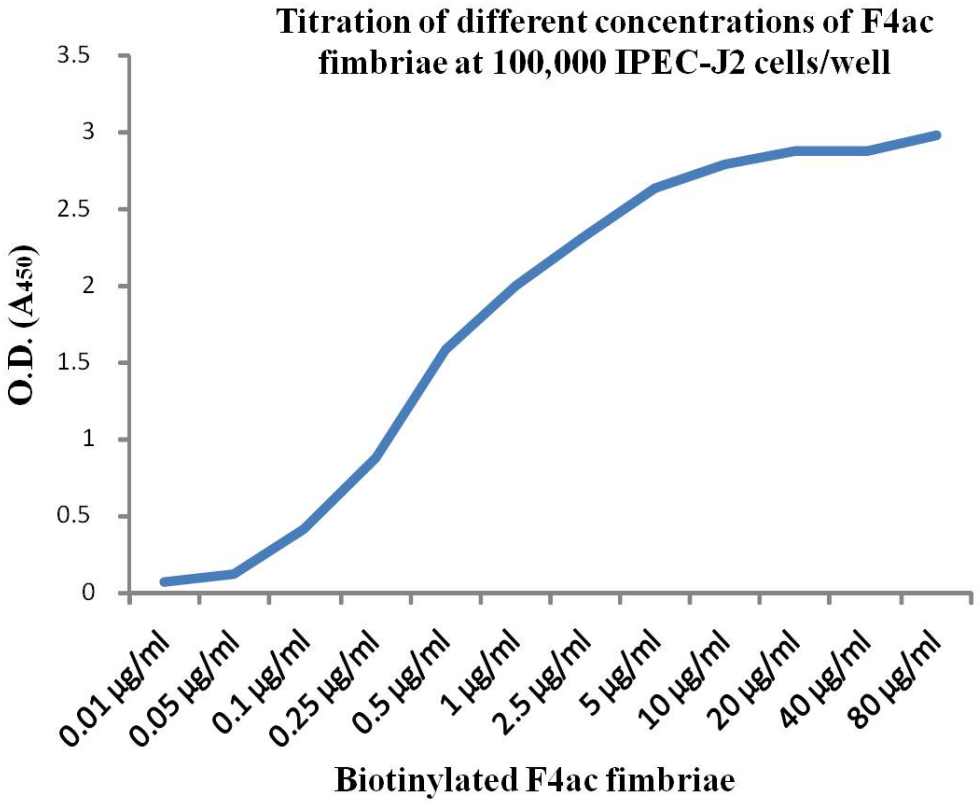

Figure 8-3 OD values obtained relating different concentration of biotinylated F4ac fimbriae to optimal number of porcine intestinal epithelial cells (IPEC-J2). Curve shows graphically the plateau maximum value of about 2.5 OD units.

The results demonstrated that concentrations of biotinylated F4ac fimbriae in a range between 0.5-1 µg/ml obtained optimal OD values (1.5-1.7 OD units). Hence it was decided to use 0.5 µg/ml of biotinylated F4ac for the purpose of this study (Chapter 5).